12-1-2011

# Role of VimA in Cell Surface Biogenesis in Porphyromonas gingivalis

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in conjunction with the
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# Role of VimA in Cell Surface Biogenesis in *Porphyromonas gingivalis*

by

Devon Osbourne Jr.

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Microbiology and Molecular Genetics

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December 2011

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

First and foremost, I thank God, for providing me with this opportunity of higher learning, and placing me in an environment of creative, balanced, Godly lab associates. During the occasions of aggravation and outright annoyance, when simple experiments refused to work, God has been a source of strength and comfort.

I would like to thank Dr. Hansel Fletcher for his guidance and mentorship. You have played an intrinsic role in helping me to be a better scientist, by providing helpful insights and asking probing questions. Most significantly, you allowed me the freedom to pursue and develop hypothesis and experiments as dictated by the Data. Your willingness to share information about the grant writing process, and other potentially difficult scientific experiences/processes has been most instructive.

To my lab associates: Dr. Antonette Robles (Nettie), Alexia Ximines (Lexi), Arun Muthiah (Mutiter), Dr. Aruni Wilson (Wilkison), Dr. Francis Roy (Frankiske), Leroy Henry (Quita), Dr. Marie-Clair Boutrin (St. Clair), Dr. Rachelle McKenzie (McBerry), and Dr. Yuetan Dou (Yuetange), thank you for making this journey an unforgettable experience. You have been great to work with.

To my committee members: Dr. Christopher Perry, Dr. Mark Johnson, Dr. Penelope Duerksen-Hughes and Dr Ubaldo Soto. Your support and helpful suggestions were truly appreciated.

Special thanks to my immediate and church family (Imani Praise Fellowship SDA). To my Mother and Father, thank you so much for your unwavering support, positivity and prayers. You have never been hesitant to give of your best, or to sacrifice for your children. To Shauna-Kay, Janice and Lavern, thank you for your encouragement and humor. To my church family, thank you for your prayers and inspiration.

You have all been a source of inspiration and kindness, and I thank God for all of you and the difference that you have made in my life.

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ABBREVIATIONS

| | |
|---|---|
| ABA | *Agaricus bisporus* lectin |
| AFM | Atomic Force Microscopy |
| ALNA | Acetyl-lysine-p-nitroanalide |
| BApNA | N-a-benzoyl-DL-arginine-p-nitroanalide |
| BHI | Brain Heart Infusion |
| ConA | Concanavalin A |
| DBA | *Dolichos biflorus* lectin |
| ECA | *Erythrina cristagalli* lectin |
| Gal | Galactose |
| GalNAc | N-acetylgalactosamine |
| Glc | Glucose |
| GlcNAc | N-acetylglucosamine |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| Kb | Kilobases |
| kDa | Kilodaltons |
| Kgp | Lys-X gingipain |
| LDS | Lithium dodecyl sulfate |
| LPA | *Limulus polyphemus* lectin |
| LPS | Lipopolysaccharide |
| MS | Mass spectrometry |
| MOPS | Morpholinepropane sulfonic acid |
| Mt-Rgp | Membrane-type Arg-X gingipain |
| ORF | Open reading frame |
| PAD | Peptidyl arginine deiminase |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PS | Polysaccharide |
| PCR | Polymerase chain reaction |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| RecA | Recombinant protein A |
| Rgp | Arg-X gingipain |
| SBA | Glycine Max lectin |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electophoresis |
| TEM | Transmission electron microscopy |
| TFA | Trifloroacetic acid |
| TLCK | N-a-p-tosyl-L-lysine chloro-methyl ketone |
| TSBKH | Trypticase soy broth containing menadione and hemin |
| VimA | Virulence modulating protein A |
| WTA | *Triticum vulgaris* lectin |

LIST OF *Porphyromonas gingivalis* STRAINS AND MUTANTS

| | |
|---|---|
| *Porphyromonas gingivalis* W83 | Wild-type, parent strain |
| *Porphyromonas gingivalis* ATCC33277 | Wild-type |
| *Porphyromonas gingivalis* 381 | Wild-type |
| *Porphyromonas gingivalis* FLL92 | *vimA*-defective mutant |
| *Porphyromonas gingivalis* FLL92D | *vimA* Complemented in FLL92 |

ABSTRACT OF THE DISSERTATION

Role of VimA in Cell Surface Biogenesis in *Porphyromonas gingivalis*

by

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Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, June 2011
Dr. Hansel M. Fletcher, Chairperson

*Porphyromonas gingivalis* is an important etiological agent of periodontal disease - a disease that affects an estimated 49,000,000 people in the United States of America. Periodontal disease includes gingivitis – inflammation of the gums, and periodontitis – destruction of the teeth and their supporting tissues. *Porphyromonas gingivalis* is associated with the chronic form of periodontal disease in addition to several systemic diseases.

The *vimA* gene of *P. gingivalis* has been previously shown to play a significant role in the biogenesis of gingipains (trypsin-like cysteine proteases). The *vimA* has also been demonstrated to play a role in hemolysis, hemagglutination, autoaggregation, post-translational protein modification, and LPS biogenesis. Though significant progress has been made in characterizing this gene, much remained unsolved prior to this work, especially as it relates to the molecular mechanisms and interactions of this protein in surface biogenesis, transport and membrane integrity. The focus of our work was to clarify the role of VimA on cell surface biogenesis. We were able to show via TEM and AFM that there were significant differences between the v*imA*-defective mutant (FLL92) and the wild-type (W83). Particularly, the topography of FLL92 showed numerous fine structures compared with W83, and had an irregular, loosely bound capsule. Fimbrial synthesis was also affected by the VimA mutation. FimA coupled immunogold particles localized with the abundant fimbrial appendages in FLL92, compared with only a few

adhering in W83. Lectins were used to clarify the effect of the *vimA* mutation on the glycosylation of outer-membrane proteins. Outer membrane proteins glycosylated with Galactose (β 1,3) N-Acetylgalactosamine, N-acetyl-α-D-galactosamine, Galactose (β 1,4) N-Acetylglucosamine, N-acetyl-D-galactosamine and Sialic Acid (N-Acetyl neuramic acid) were affected by the *vimA* mutation. Mass Spectrometric analysis of outer-membrane and extracellular proteins identified several proteins in FLL92 that were aberrantly expressed, missing and of varying abundance. In-silico analysis of VimA predicted a likely role as an acyl transferase, with structural similarities to the FemABX family of proteins – these proteins are involved in peptidoglycans synthesis. The expression profile of lipid modified $^{3}H$ labeled outer membrane proteins from FLL92 when compared with W83 was unchanged; however, a 27 kDa protein was observed in the extracellular fraction of FLL92 that was twice as abundant in FLL92 as in W83. Peptidoglycans isolated from FLL92 were shown to be dissimilar from those isolated from W83 via TEM and AFM, with a slower rate of hydrolysis when exposed to peptidoglycan specific hydrolytic enzymes. Taken together, we provide evidence that the VimA in an important regulator of membrane biogeneis, and is a putative lipid transferase.

# CHAPTER ONE

# INTRODUCTION

## Periodontal Disease

Periodontal disease is the major cause of tooth loss in U.S. adults. Over 50% of all American adults have gingivitis on an average of 3 to 4 teeth (107). This disease affects the periodontum - the supporting tissue surrounding the tooth (periodontal ligament, gingiva, and the alveolar bone). The disease results from the interaction of heterogeneous etiological factors; including social and behavioral modulations, genetic or epigenetic traits of the host, and formation of a complex subgingival biofilm (53). Periodontal disease is initiated by the accumulation of microbial plaque in the gingival crevice region; this plaque induces an inflammatory response – gingivitis (mild and reversible inflammation of the gingiva – gum). In certain individuals, this condition may progress to a chronic destructive inflammatory stage - periodontitis. This results from a complex microbial infection which leads to tissue destruction, resulting from the perturbation of the homeostasis between the host defenses and the subgingival microbiota (119). The categories of periodontal disease include: gingivitis, chronic periodontitis (or adult periodontitis), general juvenile periodontitis, aggressive periodontitis, disease related periodontitis, and acute necrotizing periodontitis (6). Periodontitis is irreversible, as it destroys bone and other tooth supporting structures.

## Risk Factors for Periodontitis

The risk factors for periodontal disease include: smoking, hormonal changes in girls/women –makes gums more sensitive and thus increases susceptibility to gingivitis,

diabetes – 30% of all diabetics are estimated to have some form of periodontal disease, genetic predisposition – 30 % of the population are 6 times more likely than the rest of the population to develop the disease (74); medications – some drugs lessen salivary flow thus affecting oral health, poor nutrition, stress, and systemic diseases. Periodontal diseases have also been implicated in the increased risk of heart attack, increased risk of preterm delivery and low weight babies, and increased difficulty in controlling blood sugar levels in people with diabetes (29).

## Bacterial Etiology of Periodontal Disease

The bacterial etiology of periodontal disease is rather complex and a variety of organisms have been implicated in the initiation and progression of the disease. The prevailing view, as supported by the literature, indicates that a number of these disease-causing microbes are part of the normal flora of a healthy mouth, existing in commensal harmony with the host amongst the 350 different bacterial species that are resident in the subgingival niche. A disease episode ensues once there is a shift in the ecological balance, due to the alteration in the absolute or relative number of certain organisms, changes in pathological potential or modulation of particular host factors (Fig. 1.1).

The progression of adult periodontal disease was initially thought of as a broad group of infections with multiple bacterial etiologies; recent evidence however has suggested a primary pathogen: *Porphyromonas gingivalis.* This organism may act either alone or in tandem with other putative periodontal pathogens including: *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Bacteroides forsythus, Campylobater rectus, Prevotella intermedia, Treponema denticola, Treponema pectinovorum, Selenomonas sputigenam, and Eikenella* as part of a mixed infection; and possibly in concert with the absence of beneficial species or the immunological deficiencies of the host (44,57,82).

In response to the bacteria accumulating on the teeth, the host mounts an inflammatory response in the gingival tissue, which seeks to remove bacterial products such as LPS and proteases that have penetrated the tissue. This inflammatory response can also activate matrix metalloproteases - agents of collagen loss in tissues. Elevated levels of interstitial collagenase often arise owing to the conversion of latent collagenolytic enzymes into their active form by proteases and reactive oxygen species. (83-85).

Commensal oral *Streptococci* are the principal early/initial colonizers of the salivary pellicle on the coronal tooth surface. Subsequent colonization of additional Gram positive along with Gram-negative bacteria occurs only as these initial colonizers are established. As subgingival plaque develops due to the extension of the biofilm below the gum line, the colonization of more pathogenic Gram-negative bacteria such as *P. gingivalis* is manifested, and marks a further stage in the disease maturation (79,130,154).

## Virulence Factors

A variety of bacterial cell surface components including: capsule, polysaccharides, proteases (gingipains), hemmaglutinin, lipopolysacharides, major outer membrane proteins and fimbriae contribute to the virulence of *Porphyromonas gingivalis* (82,155). This organism forms black-pigmented colonies on blood agar plates – due to accumulation of μ-oxo heme dimer on the cell surface (120), is Gram-negative, asaccharolytic anaerobe, which is an important etiologic agent of periodontal disease, and is also associated with other systemic illnesses including cardiovascular disease (28,31,43,98).

## Fimbriae

The fimbriae of *P. gingivalis* consists of a 41-49 kDa and 67-75 kDa cell surface protein (depending on the strain) (34,47,49,156). These proteins constitute two distinct sets of adhesins/receptors, and are recognized as critical virulence factors which influence the initiation and progression of the disease (50). They cooperatively mediate adherence of *P. gingivalis* to oral epithelial cells (61,132,138), salivary components - such as proline-rich proteins and statherin (5,8,94) and co-aggregation with other bacterial cells (4). The major (long) fimbriae are filamentous components on the cell surface. Fimbrillin (FimA) is the major subunit protein and mediates bacterial adhesion and colonization in targeted sites. Sequence diversity of the *fimA* gene has been used to classify fimbriae into six genotypes (I-V and Ib). The majority of periodontal patients carry *P. gingivalis* with type II *fimA* organisms; these are associated with the more severe forms of periodontitis followed by type IV. In healthy adults, the most prevalent *fimA* type of *P. gingivalis* is type I. When type II FimA was substituted for type I FimA in the mouse periodontitis model, there was increased phagocytosis by macrophages, suggesting that type II macrophages interact more efficiently with macrophage phagocytic receptors than type I fimbriae (148). The biofilm of Type II FimA also differs from those associated with type I, i.e. are more luxuriant and are filled with widely clumped and tall colonies compared with type I FimA (78).

### Immune Response to FimA

Human peripheral macrophages and neutrophils have been shown to be induced to overproduce several proinflammatory cytokines such as tumor necrosis factor, interleukin 1 and interleukin 6 by fimbriae (104). T-cell activation can also be induced by fimbriae in mice (60). Major fimbriae is also required for initial entry of *P. gingivalis* into osteoblasts (157).

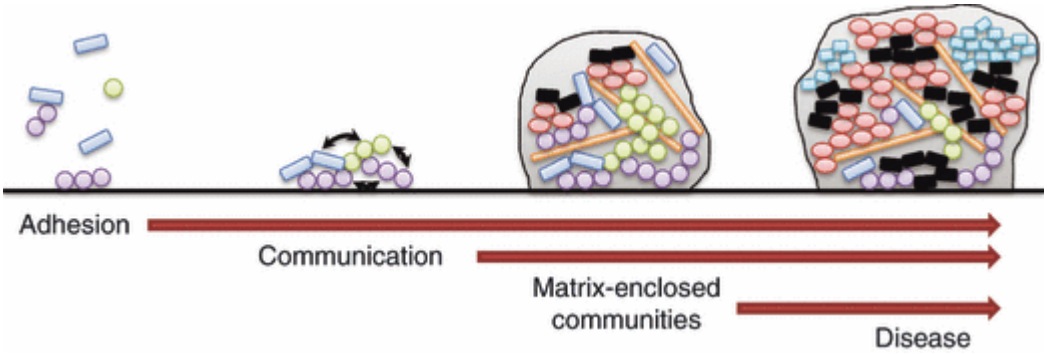


Fig 1.1. Stages in the formation of dental plaque. Colonization of tooth surfaces is initiated by bacterial adhesion to the salivary pellicle. Coaggregation and coadhesion facilitate the development of multispecies communities. The exchange of chemicals between neighboring bacteria promotes co-operation or competition. In some cases, communication does not occur until a critical biomass is reached. Adherent bacteria produce a matrix of complex carbohydrates and/or extracellular nucleic acids, which helps to bind the biofilm together and to protect the encased cells. Dental caries or periodontitis arises from a shift in the microflora and an accumulation of pathogenic bacteria (63)

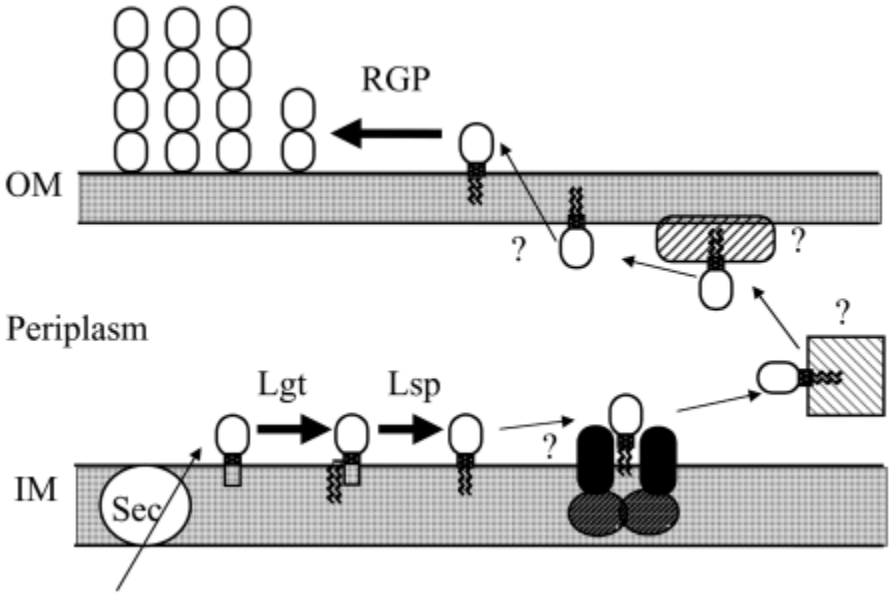


Fig 1.2. A model for the transport mechanism of the major structural proteins of *P. gingivalis* cell surface filaments. In this model, a primary form of the major structural proteins of *P. gingivalis* cell surface filaments is transported from cytoplasm to periplasm via the general secretion pathway, where the cysteine residue in the lipoprotein box is modified by diacylglyceryl transferase (Lgt). The precursor form is then cleaved at the N-side of the cysteine residue by signal peptidase II (Lsp). After the major structural proteins are transported on to the outer face of the outer membrane, they are processed further by Rgp to yield their mature forms, which subsequently assemble into filamentous structures. (124)

## FimA Regulation

Several environmental cues modulate the expression of the *fimA* gene; these include elevated temperature, hemin concentrations, cell density, serum and salivary concentrations (150). There is also evidence to suggest that the FimA can positively regulate its own expression (151) through a two-component system which involves the *fimS* and *fimR*, suggesting multiple mechanisms of regulation control (152).

At the N-termini of the primary translated forms of fimbrillin and the 75 kDa protein is a 10 (34,153) or 46 and 49 amino acid long peptide extension respectively (70,108), which is cleaved by arginine specific cysteine proteinase (Rgp). In all six *fimA* genotypes, the first 27 amino acids are the same, suggesting that the prosequence of the primary translated protein of *fimA* is processed sequentially by several peptidases (97). The maturation and translocation of fimbrillin and the 75 kDa protein are dependent on Signal peptidase II, these proteins are transported to the outer membrane by the lipoprotein sorting system, the mature form of FimA is then autopolymerized to form fimbriae (124,126) Fig. 1.2.

## Mfa Structure and Immunogenicity

An additional interaction is required for *P. gingivalis* to accrete to the mixed species biofilm. The short fimbriae (*mfaI*) provides this interaction (124). When viewed under the electron microscope, these fimbriae were distinctly short – 5 nm in width and 0.1 to 0.5 μm in length, compared to the FimA which is 4.6 nm in width and 1.3 μm in length (131). The subunit protein of minor fimbriae (Mfa1) is encoded by the mfa1 gene (Fig. 1.3.), and the antigenicity and size of the resulting product is different from FimA (47,138). The ability of minor fimbriae to elicit an immune response has been demonstrated in its ability to induce IL-1$\alpha$, IL-$\beta$, IL-6, and TNF-$\alpha$ cytokine expression in

mouse peritoneal (48). Studies with knockouts of *fimA*, *mfa* and *mfa:fimA* inoculated in the oral cavities of rats, showed significant suppression of periodontal bone loss when compared to the *P. gingivalis* wild-type ATCC 33277 using the *mfa1* and *mfa:fimA* knockouts. More significant bone loss was observed in the *fimA* knockout than in the *mfa* knockout; additionally, there was no auto-aggregation in the *fimA* knockout and markedly lower levels of adherence to single cells, whereas the double knockout completely lost its adhesive capabilities (138).

The Mfa1 molecule has been shown to be the same as the 75 kDa outer membrane protein, the 67 kDa major outer membrane protein, and pg-II (a 72 kDa cell surface protein) reported in several studies. Another minor fimbriae has been isolated from strain 381 (9) which in contrast to 33277 is 53 kDa and has no immunological cross reactivity to the 67 kDa minor fimbriae from 33277 (7). Mfa1 has also been shown to be a receptor for SspB (Streptococcal surface polypeptide) BAR, which is necessary for optimal coadhesion between *P. gingivalis* and *S. goronii.* Antibodies to this protein and recombinant Mfa1, competitively inhibited *P. gingivalis – S .gordonii* binding; additionally, recombinant Mfa1 was found to bind SspB BAR peptide in a dose dependent manner (112). The degree of *P. gingivalis* aggregation was found to correlate with the expression levels of *mfa1* and *fimA.* In particular, the down-regulation of *fimA* and the up-regulation of *mfa1* are believed to bring about autoaggregation of *P. gingivalis*; additionally, minor fimbriae appears to play an important role in microcolony formation by facilitating cell-cell interactions and the maturation of monospecies biofilms (88).

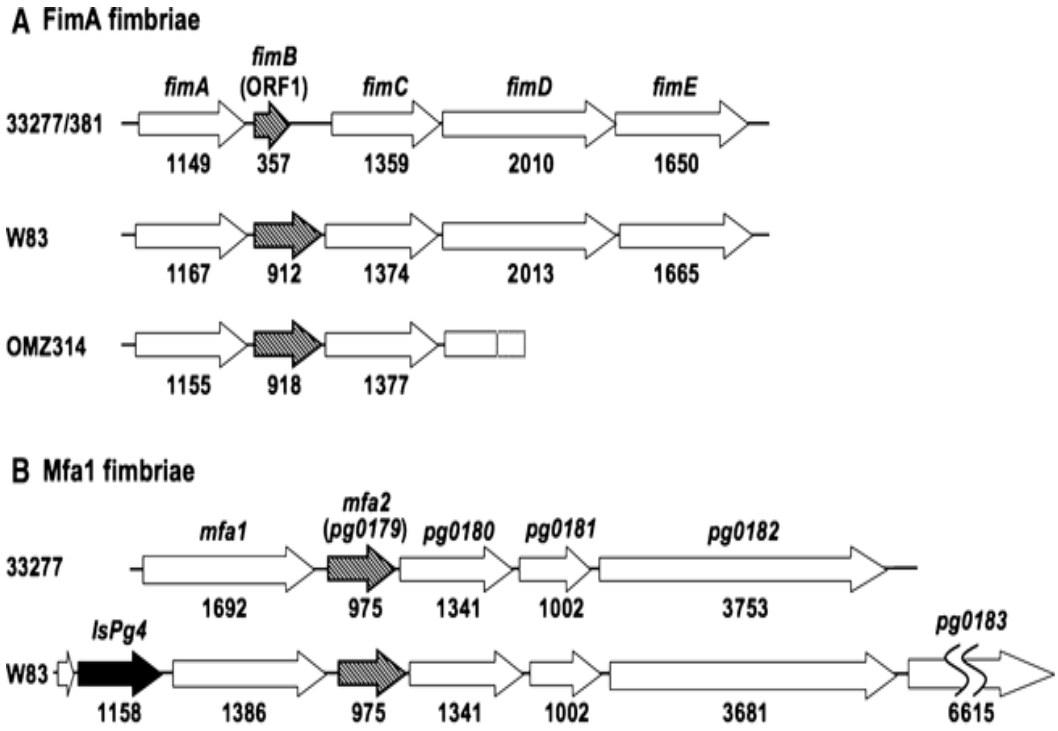


Fig 1.3. Schematic diagrams of the downstream genes of *fimA* (A) and *mfa1* (B) in limited strains. (A) The top line shows the FimA gene cluster of *Porphyromonas gingivalis* ATCC 33277 and 381 (*fimA* genotype I). Below are *P. gingivalis* W83 (genotype IV) and OMZ314 (genotype II). Open-reading frames are represented by arrows indicating their 3′ ends. The *fimA* sequences of ATCC 33277, W83 and OMZ314 were obtained from GenBank (D42067) and TIGR GenBank (AB261607), respectively. The downstream sequence of *fimD* in OMZ314 is indicated by a box with a broken line; the sequence further downstream is not yet available. W83 does not produce FimA fimbriae, partly because of a defect in FimSR, a two-component signal transduction system, positively regulating their production. (B) A potential *mfa1* gene cluster in ATCC 33277 and W83. The gene cluster appears to be composed of at least five genes, including *mfa1*, with the same transcriptional direction except that *mfa*1 is split into pg0176 and pg0178 by ISPg4 (pg0177), indicated by the solid arrow, in W83. Therefore, W83 does not produce Mfa1 fimbriae either. The gene *fimB* (and its corresponding genes) and *mfa2* (and its corresponding gene) are highlighted as hatched arrows in FimA and Mfa1 gene clusters, respectively. The number values shown under the genes indicate their lengths in bp (155).

## Hemagglutinin

Hemagglutination in *P. gingivalis* is a distinctive property, which differentiates it from other asaccharolytic, black pigmented anaerobes, and is essential for its growth (99). Hemagglutination is related to the ability of the bacteria to adhere to host cells, and is particularly significant since *P. gingivalis* grows faster in cultures with hemin than in cultures without hemin (118). Hemin accumulation by *P. gingivalis* directly affects its pathogenicity, exemplified by the decrease in transcripts of hemagglutinating genes (86) gingipain enzyme activity (73), and promoter activity of fimbriae gene (150) under hemin-limited growth conditions. There are several reported genes that have been demonstrated to encode hemagglutinin domains, these included: *rgpA(HagE)*, *kgp,* (30,100) , *hagA*, *hagB*, *hagC* (86) and *hbp35* (55,56,136). Mutants of *rgpA*, *kgp* and *hagA*, showed decreased abilities to agglutinate erythrocytes, while an *rgpA-kgp-hagA* triple mutant had no hemagglutinating activity (100,123).

### Hemagglutinin Domains

The adhesion domain coded for by *hagA* is similar to that encoded for by the carboxy terminal regions of *rgpA* and *kgp.* These adhesin domains are involved in cell induced hemagglutination as well as platelet aggregation (96,118). Conversely, *hagB* and *hagC* share over 90% homology, in contrast to only 40% with their upstream and downstream regions (86). HA-Ag2 a cell bound hemagglutinating adhesin, was identified as one of the common antigens of the species; H1 of HA-AG2 is hemmaglutinin specific while the F2 domain is fimbriae specific (17). The recombinant form of Hgp44 located between Ser720 and Arg1138 of RgpA, and present in Kgp and HagA , was demonstrated to possess the ability to agglutinate erythrocytes, this residue has multiple domains including Hgp44B which induces hemagglutination, and Hgp44A which likely functions as a suppressor of hemagglutination – also has the hemagglutinating epitope

(GVRSPEAIRGRIQGWRQKT) (100) Fig. 1.4. The Hgp44 domains of HagA, RgpA and Kgp have also been shown to be key adhesion factors for the coaggregation between *P. gingivalis* and *T. denticola* (62). Using phase displayed epitope mapping, a functional domain of hemagglutination - IALDQTLGIP - was identified in HBP35. The mutant of HPP35 was non black pigmented, showed decreased autoaggregation and coaggregation with other bacteria, and had reduced gingipain activity(55).

## Hemagglutinin Immunogenicity

Immunoglobulin G (IgG) antibody was demonstrated to react with recombinant *P. gingivalis* hemagglutinin with both chronic and generalized aggressive periodontitis patients. Most of this IgG belonged to the IgG1 and IgG3 subclasses. Interestingly, there was no relationship with measures of periodontal attachment loss (15). Other studies have demonstrated that IgG2a and IgG2b, both of which are complement fixing are the predominant IgG subclass induced by HagB (76). HagB has been demonstrated to adhere to Human Coronary Artery Endothelial Cells (HCAE) (133), the purified form has also been found to elicit a protective immune response in the rat bone loss model (72). Immunization with Hgp44 also provided protective effect against *P. gingivalis* induced alveolar bone loss (93). Nasal immunization with the antigenic region of HagA fused to a Maltose-Binding protein in mice was effective in eliciting IL-4 and IL-5 producing Th2-type $CD4^+$ T cells for the induction of serum IgA, IgG, and mucosal IgA antibody responses. These specific immune responses provided protective immunity against alveolar bone loss caused by *P. gingivalis* infection (36).

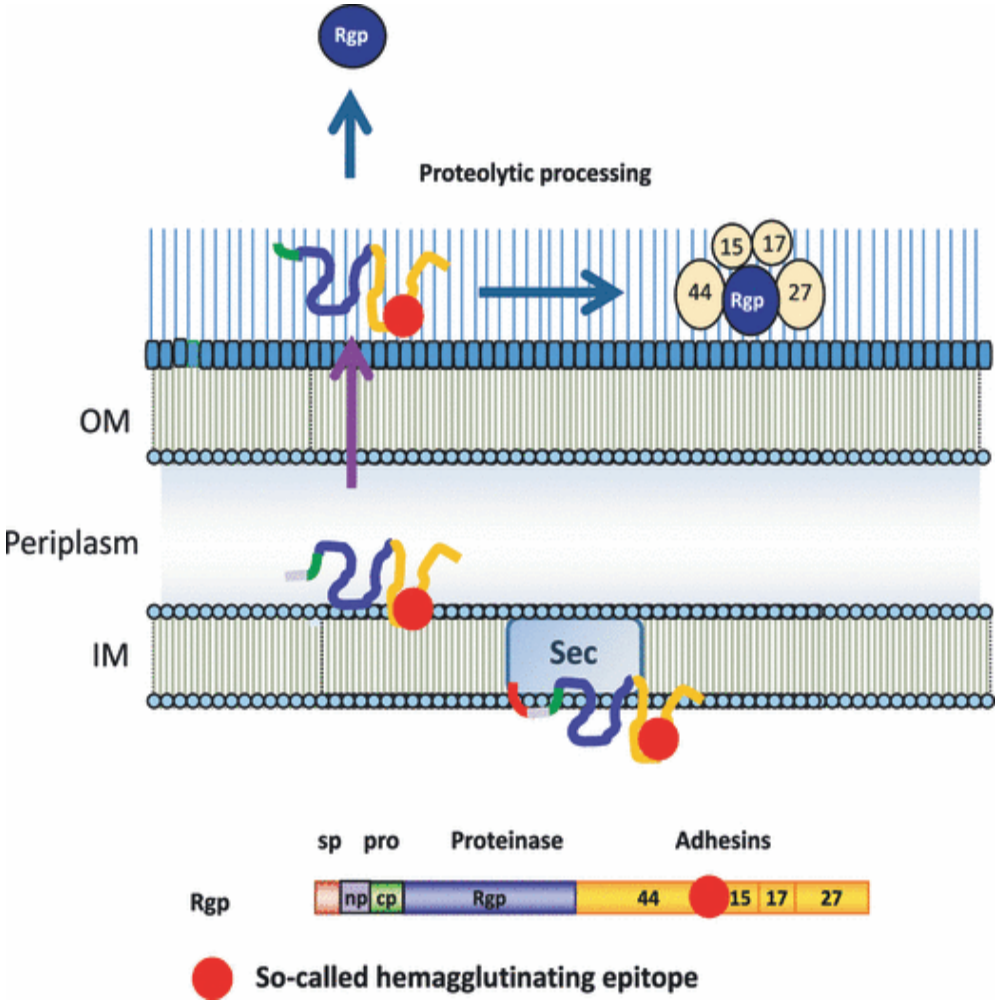


Fig 1.4. Inhibitory effect of the so-called hemagglutinating epitope on the hemagglutinating activity of Hgp44 during transport of gingipain proproteins. Gingipain proproteins are transported from the cytoplasm to the periplasm by the Sec-dependent pathway and secreted from the periplasm to the cell surface by the Por secretion system. (The so-called hemagglutinating epitope is removed from the proproteins after translocation to the cell surface. cp, C-terminal portion of prosequence; IM, inner membrane; np, N-terminal portion of prosequence; OM, outer membrane; pro, prosequence; sp, signal peptide (99).

## Lipopolysaccharides

The lipopolysaccharides of *P. gingivalis,* were initially demonstrated to have significantly different low endotoxic properties when compared to enterobacterial LPS (95), and it was postulated that this difference in endotoxic potency was dependent on the chemical structure of it's lipid A. Compositional analysis of the LPS revealed that the polysaccharide component consisted of rhamnose, glucose, galactose, mannose, glucosamine and galactosamine (14). *P. gingivalis* has a novel lipid A structure when compared to enterobacterial and *B .fragilis* lipid A. In particular, the backbone of this lipid A was found to consist of a phosphorylated β-(1-6) linked glucosamine disaccharide at the 1 position of the reducing sugar, additionally the structure lacked an ester linked phosphate group bound to the hydroxy group at the 4' position of the nonreducing sugar. This unique structure was believed to contribute to the mitogenic effects noted on splenocytes in $C_3H$/HeN and $C_3H$/HeJ mice (103). LPS was further demonstrated to be comprised of two macromolecules containing different glycan repeating units: O-LPS (with O-antigen tetrasaccharide repeating units) and A-LPS (with anionic polysaccharide repeating units) (110).

### O LPS

The core oligosaccharide (OS) of O-LPS occurs in two glycoforms: a capped core containing the site of O polysaccharide attachment and an uncapped form devoid of O polysaccharide. This core OS of *P. gingivalis* O-LPS is a highly unusual structure (109). The O antigen polymerase (OS1142) was shown to differ from the O antigen ligase (OS1051) by up to 5 additional α-(1→3)-linked mannose residues attached to the nonreducing terminal residue of α-mannose at position 4 of the main outer core glycoform, which forming a capped core. It is believed that the actions of α-1,3 –

mannosidase (PG1711) may be important for generating the uncapped core from the capped core or alternately a manosyltransferase (PG0129) may add additional α-1,3 linked Man residues to the shorter uncapped core to generate the capped core (109).

## A-LPS

The APS is comprised of a phosphorylated branched D-Man-containing oligomer composed of an $\alpha 1 \rightarrow 6$- linked D mannose backbone to which $\alpha 1 \rightarrow 2$ linked D-Man side chains of different lengths at position 3 are attached. It has been suggested that the synthesis of APS occurs via a wzy (polymerase)-dependent pathway in which polymerization of repeating units formed on the cytoplasmic face of the inner membrane, occurs after they are transported or flipped across the cytoplasmic membrane to the periplasmic face. PG1142 has been shown to be the sole polymerase capable of polymerizing the O-antigen repeating unit in *P. gingivalis* (111). The final step in the assembly of these two different macromolecules is catalyzed by a relaxed-specificity ligase named WaaL, which is able to attach either a tetrasaccharide repeating unit or a branched phosphorylated mannan to the lipid A core (114). Recombinant Kgp-RgpB-Myc proteins were shown to bind to the LPS fraction of wild-type *P. gingivalis* in vitro, suggesting that gingipains may be anchored to the cell surface through surface polysaccharides (120).

## LPS Maturation

Several genes have been implicated as being involved in the formation of extracellular polysaccharides and glycan additions of gingipain-adhesin complexes – *vimA*, *vimE*, *vimF*, *porR*, *rfa* and *ugdA* (105,110,120,125,141-143) these mutants lose colonial pigmentation and show no reactivity to MAb 1B5 (monoclonal antibody which

react specifically with the modification Manα1-2Manα1-phosphate side chain) (109). Increased sensitivity to serum killing was observed in APS biosynthesis defective mutant strains (128). LPS was also found in great abundance in membrane vesicles, these packaged LPS were capable of penetrating periodontal tissue and eliciting a destructive host induced immune response (46,92). There is evidence to suggest that LPS maturation may be linked to a novel PorS secretion system, which includes the Por family of proteins in addition to LptO which directly effects lipid A structure through deacylation (18) Fig. 1.5.

### LPS Immunogenicity

*P. gingivalis* LPS has also been shown to activate human monocytes by a CD14-dependent mechanism, it has also been demonstrated to activate both human and mouse TLR2 and TLR2 plus TLR1, as well as TLR4 in transiently transfected cells expressing mCD14 (25). TLR2–mediated cell activation is believed to occur through a lipoprotein from the LPS (51). In endothelial and oral epithelial cells challenged with commercially prepared LPS, several cytokines were produced, in particular: IL-$\alpha$ and IL-6 in addition to molecules involved in the adhesion of leukocytes to inflammatory sites: IP-10, MIP-1$\alpha$, $\beta$, $\delta$ ICAM-1 and M-CSF (75).

## Gingipains

*P. gingivalis* produces a novel class of cysteine proteases termed Arg-gingipain/Arg-X (Rgp) and Lys-gingipain/Lys-X (Kgp) on the basis of their peptide cleavage specificity at arginine or lysine residues respectively (113). These enzymes are virulence factors that serve to disrupt the normal host defense mechanism in addition to their proteolytic destruction of host connective-tissue proteins, including collagen (type I

and IV) and extracellular matrix proteins such as fibronectin and laminin (3,12,71,129,134) (Fig. 1.6.). Arg-X is encoded by two separate genes (*rgpA* and *rgpB*), while Lys-X is encoded by a single gene (*kgp*) (101).

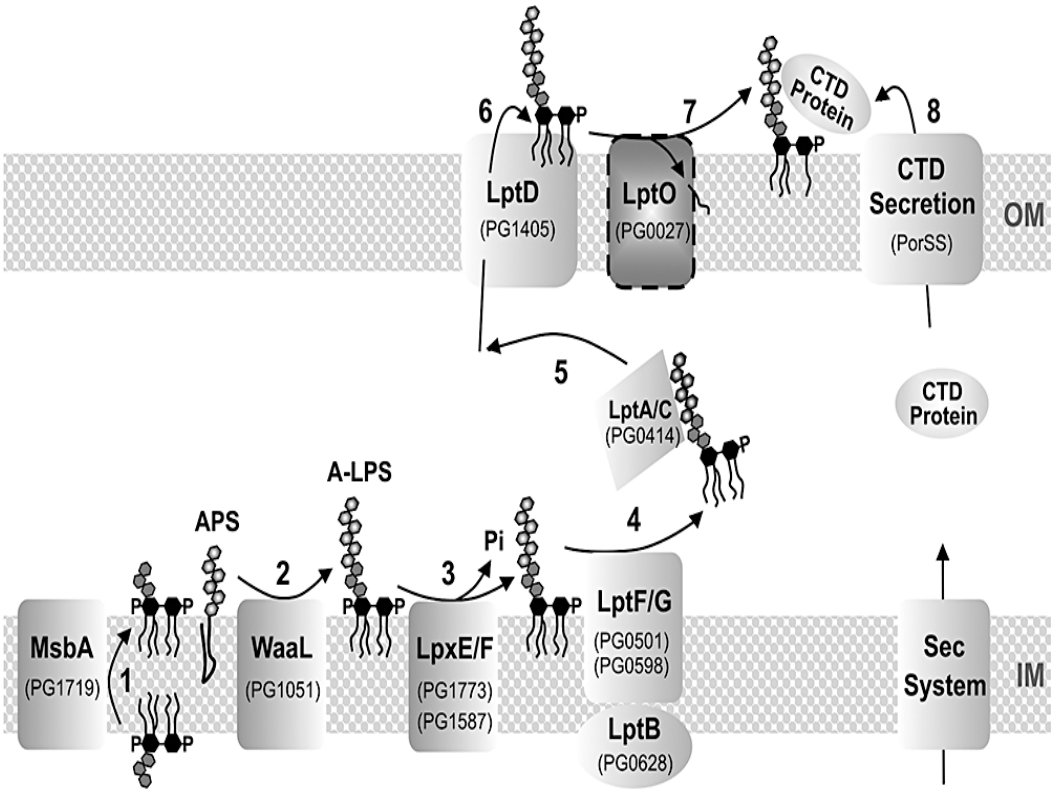


Fig. 1.5. Proposed role of LptO in a model of the co-ordinated secretion and attachment of A-LPS and CTD proteins at the cell surface. In this model it is proposed that lipid A is synthesized in the cytoplasm and linked to the core oligosaccharide. It is then flipped across the IM by MsbA (PG1719) (Step 1). The WaaL ligase (PG1051) then attaches either O-antigen or APS (Step 2). The newly formed LPS is dephosphorylated by lipid A phosphatase (PG1587 or PG1773) located in the IM (Step 3). The Lpt IM complex (PG0628, PG0501 and possibly PG0598) detaches the LPS from the IM, and may pass it to a periplasmic carrier (possibly PG0414) (Step 4), which is thought to be LptA in E. coli. The Lpt OM complex including PG1405 the putative LptD (Step 5) flips A-LPS to the outer surface of the OM (Step 6) where it can be deacylated by LptO (PG0027) with the released fatty acid chain recovered by lateral diffusion through the pore of LptO (Step 7). The deacylated A-LPS is inserted into the OM and conjugated to secreted CTD protein in a co-ordinated secretion event that involves PorSS (Step 8). (18)

## Different Forms of Gingipains

Three enzyme species make up the extracellular Arg-x protease activity in *P. gingivalis* W50 – hRrgpA (heterodimer in which the catalytic chain is noncovalently associated with an adhesin chain, 54 kDa), RgpA (free monomeric catalytic chain) and mtRgpA (membrane type RgpA which is exclusively identified with the membrane fraction, 70-80 kDa). RgpB lacks the adhesin coding region of the adhesin chain of RgpA, and closely resembles the monomeric protease derived from *rgpA*. RgpB is found in two forms – RgpB and mtRgpB (21,37,38,115). RgpA and mt-RgpA were demonstrated to be glycoproteins, isoforms of RgpA and RgpB were found to react differently with MAb 1B5, in particular RgpA, mt-RgpA and RgpB reacted strongly with MAb, while no reactivity was observed with HrgpA or RgpB. RgpA was also demonstrated to possess 20 – 30% (by weight) carbohydrates (23). RgpA was shown to contain several sugars of varying abundance including: Ara, Rha, Fuc, Man, Gal, Glc, GalN(Ac), GlcN(Ac) and N-acetylneuraminic acid (NANA) which totaled 14.4% by weight of protein, while HrgpA totaled 2.1% by weight of protein (Table 1.1.). RgpB isolated from *P. gingivalis* W50 *rgpA* isogenic mutant was found to contain Ara, Rha, Fuc, Gal, Glc, GlcA and GlcN(Ac) which totaled 10% by weight of protein (23).

## Role of Gingipains in the Host Cellular Response

Gingipains are involved in several cellular processes (80) including: growth, inactivation of cytokines and their receptors, platelet aggregation, attenuation of neutrophil antibacterial activities, increasing vascular permeability and apoptosis of gingival epithelial cells (135). It has also been suggested that protease activity may play

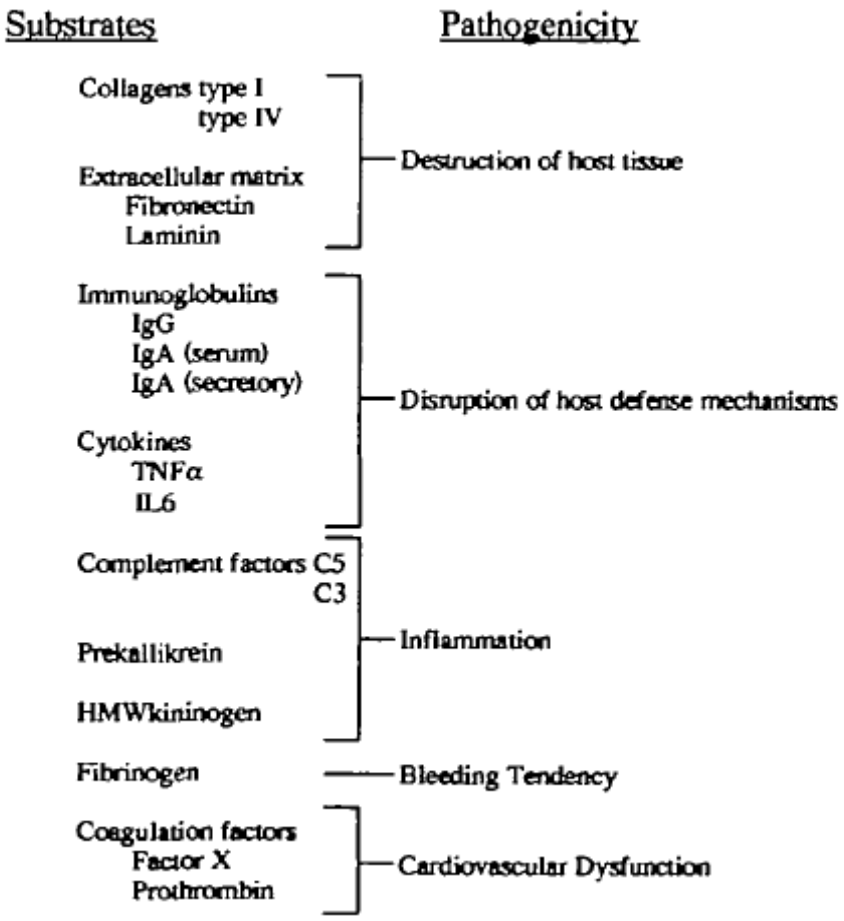


Fig. 1.6. Native proteins degraded by Rgp and Kgp and the relation of degradation to pathogenicity (69).

a role in oxidative stress resistance, hemoglobin binding, absorption and heme accumulation (2,44).

The ability of gingipains to disrupt the host defense mechanism is clearly demonstrated by their ability to degrade and inactivate immunoglobulins such as IgG, IgA and secretory IgA and cytokines including TNF$\alpha$ and interleukin 6 (10,16). The generation of radical oxygen species from activated polymorphonuclear leukocytes is also inhibited by Rgp and Kgp, suggesting that these proteinases can impair cellular components of the defense mechanisms (3,71).

## Structure of Gingipains

Based on the similarity of the nucleotide sequences, the *rgpA* and *kgp* genes can be divided into six DNA regions. Little similarity is observed between *kgp* and *rgpA* in the P region, which includes the prepropeptide and most of the proteinase domain. The CP region follows the P region and corresponds to the carboxy terminal region of the proteinase domain. There is 94% identity between *rgpA* and *kgp* in this region. There is also close homology between the A2 and A4 regions that cover most parts of the carboxy-terminal adhesin domain and shows close similarity (98 and 96% respectively). The presence of several nucleotide repeats in the carboxy-terminal domain is believed to facilitate rearrangements (Fig. 1.7.) (69).

The inability of MAb 1B5 to recognize C terminally truncated RgpB (48 to 50 kDa) (22,40), led to the suggestion that the carbohydrate modification recognized by the MAb 1B5 is in the C terminal domain, and may play a role in the outer membrane attachment of the enzyme (145). Numerous outer membrane proteins were found to have primary and secondary structure similarity to the C-terminal domain of RgpB, and were designated as CTD family; members of this family are attached to the cell surface

through cell envelope glycans, which are important for proper folding and processing in order to produce a fully functional enzyme (122).

### Gingipains as Processing Enzymes

Arg-X was found to be a major processing enzyme of several outer membrane proteins including fimbrillin, as it was able to convert prefimbrillin to the mature fimbrillin in vitro, interestingly the Lys-X was also shown to be abnormally processed in the Rgp null mutant, suggesting that Arg-X may be involved in the maturation process of Lys-X (70). There is also evidence to suggest that Arg-X is induced under iron poor conditions but down regulated under iron rich conditions (137).

## Capsule

Encapsulated *P. gingivalis* has been demonstrated to cause serious forms of infection; and mice challenged with encapsulated *P. gingivalis* develop more severe infections than those challenged with unencapsulated strains (81,139). Capsular polysaccharides (CPS) (or K-antigens) consists of several sugars including: galactosamine, glucosamine, glucose, galactosaminuronic acid, mannuronic

Table 1.1. GC-MS analysis of O-TMS ethers of methyl glycosides showing the molar ratios of monosaccharides in the oligosaccharides of HRgpA, RgpA, and LPS of P. gingivalis W50 after methanolysis (23).

| Oligosaccharide | Molar ratio | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ara | Rha | Fuc | Man | Gal | Glc | GalN(Ac) | GlcN(Ac) | NANA |
| HRgpA | | 1 | | 3.6 | 1.7 | 3 | 2 | 1 | |
| RgpA | 2.4 | 1.8 | 2.8 | 1 | 11.8 | 4 | 18 | 6.4 | 15 |
| LPS[a] | | 1 | | | 2.4 | 5 | 1.8 | 1 | |
| Core region of LPS[b] | | 1 | | | 2.5 | 3.7 | | | |

[a] Two unidentified sugars are also present.
[b] One unidentified sugar (which is also present in LPS).

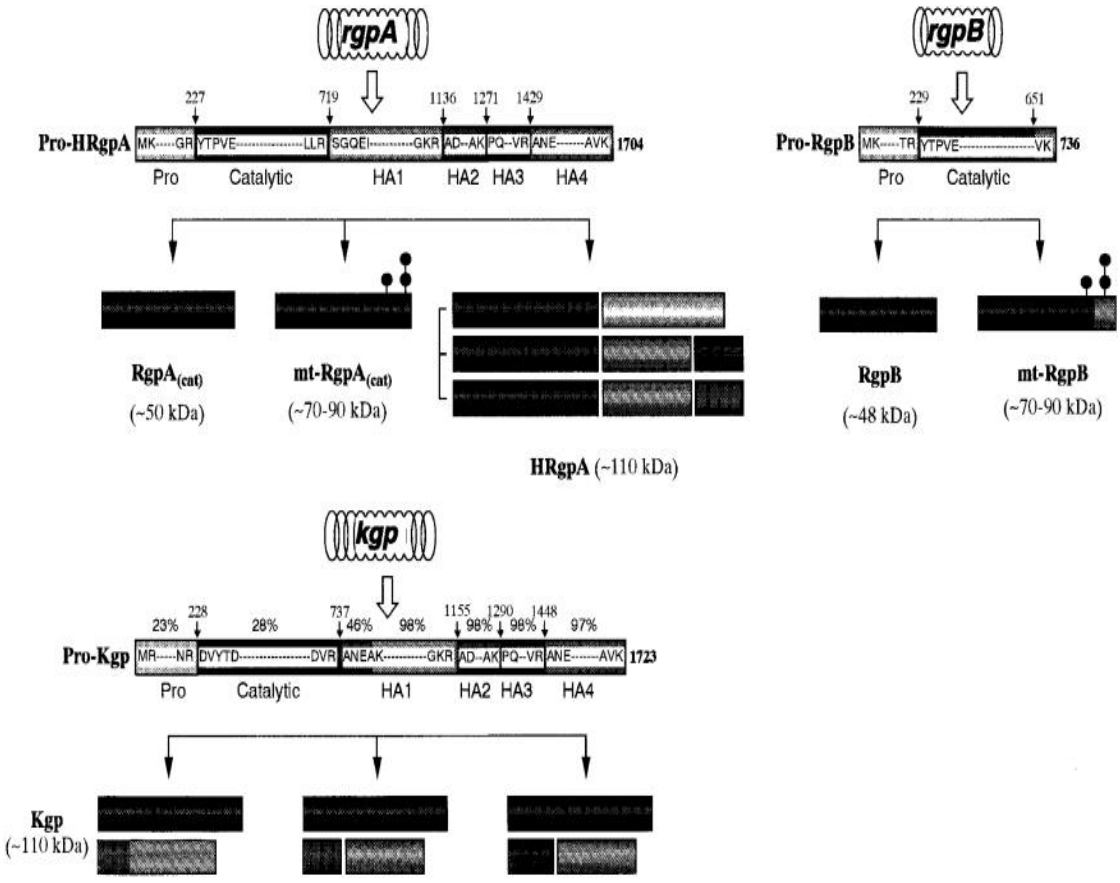


Fig. 1.7. Isoform construction by proteolytic processing and assembly of the translated products of rgpA, rgpB, and kgp genes (HG66). Open arrows denote translation of gingipain genes to their proforms. Pro, profragment; Catalytic, catalytic domatin; HA1, HA2, HA3, HA3/4, HA4, hemmaglutinin/adhesin subdomains. Inistial and end amino acids of domains are shown. Small numbered arrows denote the cleavage site amino acid numbers and large arrows denote proteolytic processing and assembly of the mature enzyme complex, respectively. The domains with high homology are shown in the same pattern and the percentage above Pro-Kgp columns denotes homology percentage of the Pro-Kgp domains for the corresponding Pro-HRgpA domains. Dots attached to mt-RgpA and mt-RgpB denote polysaccharide chains. Molecular weights of isoforms are shown in parentheses. (Modified from Potempa et al. (59)

acid, glucoronic acid and 2 acetamido-2-deoxy-D-glucose (41,121). Six serotypes have been identified in *P. gingivalis* (K1 to K6) in addition to several strains that are $K^-$ (devoid of a capsule). Adhesion of *P. gingivalis* to human pocket epithelial cells depends on the hydrophobicity of the bacteria, which is lowered by the presence of a capsule. Cells deficient in capsule have a higher adherence to epithelial cells and show increased auto-aggregation, and are usually fimbriated - show peritrichous fimbriae on their surface in contrast to the poorly adherent strains, which have significantly less fimbriae and are shorter than well adhering strains (35,77,139,149). There are significant variations between all six serotypes and limited conservation as revealed by restriction fragment length polymorphism analysis. DNA sequence analysis of the K-antigen locus of 381 which is syntenic with W83 revealed that *pg0109-pg0114* may be important for capsular expression (39). It was later determined that several essential genes including *pg0106* as well as HU (small, basic, heat stable DNA binding protein) are also necessary capsular expression (Fig. 1.8.). Interruption of *pg0106* prevented capsular polysaccharide synthesis as well as attachment and initiation of biofilm formation in vitro (11,29).

## Immunogenicity of the Capsule

CPS from *P. gingivalis* serotype K5 are able to bind *F. nucleatum* cells, and are able to inhibit coaggregation between these two oral bacteria (116), while K1 capsular polysaccharides have been shown to elicit chemokine production from phagocytic cells and may be important for establishing the inflammatory lesion observed during periodontal disease (24). All serotypes of *P. gingivalis* were able to induce a T-helper type 1 (Th1) pattern of cytokine expression. Dentritic cells (DCs) stimulated by K1 and K2, expressed higher levels of IL-1β, IL-6, IL-12p35, IL-12p40, and IFN-γ at lower multiplicity of infection (MOI) than DCs stimulated with other strains (146). Vaccination

with *P. gingivalis* CPS in the murine model prevented oral bone loss elicited by *P. gingivalis* oral challenge (45).

## Heme Transport

Iron in the form of hemin is one of the growth requirements for *P. gingivalis*, this organism is able to metabolize a broad range of hemin substrates including methemoglobin, myoglobin lactoperoxidase, and catalase (13). Owing to the toxic nature of unregulated iron, i.e. is a cofactor for the generation of oxygen radicals from peroxide, which can contribute to the oxidative stress, encountered by the bacteria; levels of iron are tightly regulated in bacteria, to provide adequate iron for bacterial growth while limiting its availability for interaction with peroxide. *P. gingivalis* does not utilize the protoporphyrin IX synthetic pathway, rather it uses hemin as a cofactor for fumarate reductase and cytochromes It must therefore acquire hemin from its environment (106). Several uptake pathways are utilized for hemin uptake; these include the htr/tlr (127), *hmu* (87) *iht (27)*, and a ferrous uptake transporter – FeoB (26,102).

### Htr/Tlr Transporter

The htr/tlr locus consists of 5 genes – *htrA* (periplasmic binding protein), *htrB* (permease), *htc* (ATP binding protein), *htrD* (unknown function) and *tlr* (TonB linked receptor). The *htrABCD* have sequence similarity to heme transport systems of other bacteria while the tlr gene has been demonstrated to be essential for growth at low hemin concentrations. A preprotein is encoded by the *tlr* gene. At the N terminus of this Tlr protein is a TonB Box I and Box III motif, these are typical of TonB linked outer membrane receptors in gram negative bacteria (127).

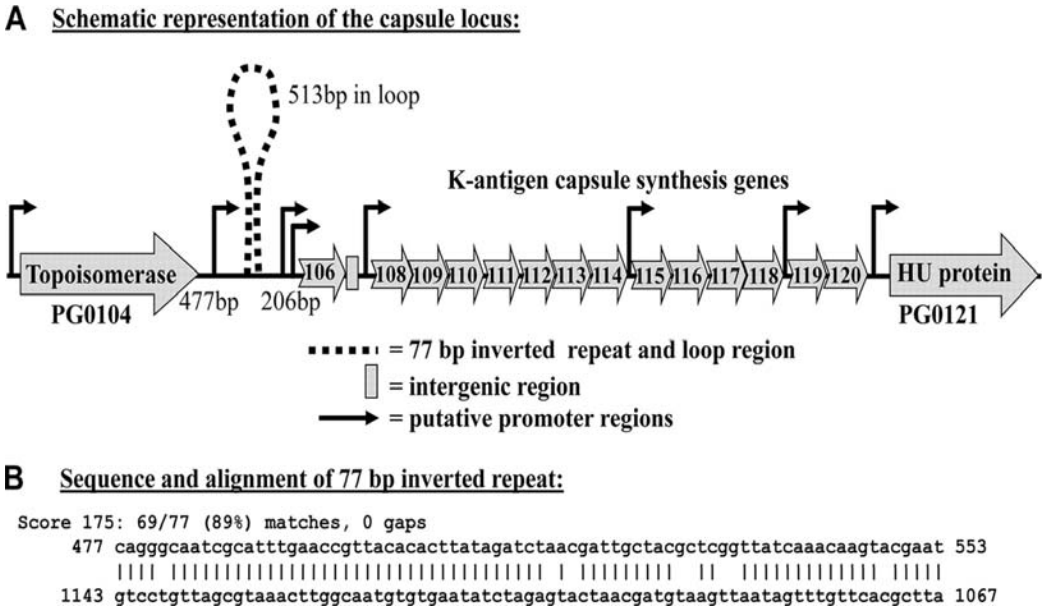


Fig. 1.8. Schematic representation of the capsule locus. (A) The position of the predicted hairpin structure created by base pairing of the 77-bp inverted repeat is indicated as a segmented line, and predicted promoter regions are shown with black arrows. The number of base pairs on either side of the hairpin and those contained within the loop are indicated. The ORF numbers correspond to TIGR CMR identifications. (B) Sequence and alignment of the 77-bp inverted repeat (11).

## Hmu Transporter

The *hmu* locus is composed of six genes, *hmuYRSTUV*; these encode a novel hybrid haemin-uptake system. Notably, *hmuY* encodes a 23kDa iron-repressible outer-membrane protein which is upregulated in iron depleted conditions in addition to the *hmuSTUV* portion which consists of a putative cobalamin biosynthesis (CobN)/magnesium chelatase (HmuS), two putative proteins with predicted transmembrane domains (HmuT and HmuU) (87).

## Iht transport

The *iht* (iron heme transport) locus is comprised of five genes – *ihtA* (TonB linked receptor), *ihtB* (accessory lipoprotein), *ihtC* (periplasmic binding protein), *ihtD* (permease) and *ihtE* (ATP binding protein) iron heme transport). IhtB has sequence similarity to the CbiK of *S.typhimurium,* which is an anaerobic cobalt chelatase. IhtB contains 13 of the 14 conserved residues that are present in the PPIX ferrochelatase anaerobic cobalt chelatase class, and has active site residues typical of chelatases. A substantially increased lag time was observed with heme limited *P. gingivalis* incubated with heat inactivated IhtB peptide-specific antiserum prior to inoculation in a medium containing hemin. This supported the view that IhtB is involved in iron transport into the cell (27).

## FeoB1 Transporter

*P. gingivalis* has two *feoB* genes – *feoB1* is monocistronic and encodes a protein of 844 amino acids, the second *feoB2* encodes a 725 amino acids. Both proteins share over 30% identity and 50% similarity with *E.coli* FeoB, and their genes were transcribed under excess heme condition with a second gene, *pg1296* and *mntR*, respectively.

Whereas inactivated *feoB2* had no effect on radiolabeled iron uptake, *feob1* inactivation abolished the transport of radiolabeled iron and resulted in a 2-fold decrease in cellular iron, when grown under excess heme conditions. Additionally, inactivated feob1 in a murine lesion model of infection rendered the bacterium avirulent, in contrast to the feob2 mutation, which induced lesions with no significant difference in size (26).

## Oxidative Stress

*P. gingivalis* possesses a low tolerance to oxygen when compared to other anaerobes (33), though incapable of colony formation on plates incubated aerobically, it can tolerate aerobic conditions and can grow in the presence of oxygen at concentrations lower than in air. The presence of a heme rich environment increases its aerotolerance. When *P. gingivalis* was grown under an oxygenated environment, there were accompanying changes in cell morpholgy – bacillary when stressed as opposed to coccoid, antioxidant enzymes – NADH oxidase, NADH peroxidase and SOD increased under oxygenated conditions, proteinase activity – Rgp whole cell activity increased while Kgp whole cell activity decreased, and fermentation end-products (32).

The FeoB1 transporter was demonstrated to impart peroxide sensitivity to microbial cells. Cells deficient in FeoB1 showed a 40% to 70% reduction in their ability to grow in the presence of peroxide (102). Similarly in knockouts of the aphC (alkyl hydroperoxide reductase) which has peroxide scavenging activity, the mutant showed increased sensitivity to hydrogen peroxide, but exhibited no change in its pathogenic potential (65). *P. gingivalis* produces an Rbr-like protein (rubrerythin) which is induced above its constitutive anaerobic level in response to dioxygen or hydrogen peroxide. Strains with disrupted *rbr* showed increased oxidant sensitivity (33). SOD (superoxide dismutase) expression is dependent on specific environmental conditions; these include increased temperature, oxygen and alkaline pH. Though this enzyme is crucial to the

survival of the organism in aerobic environments as well as conditions generated by specific reactive oxygen species (ROS), it does not protect the organism against exogenously generated superoxide or human neutrophils (91).

## The *recA* Locus

### BCP Plays a Role in Oxidative Stress

The *recA* locus consist of 6 putative genes – *bcp* (0.5 kb), *recA* (1 kb), *vimA* (0.9 kb), *vimE* (1.3 kb), *vimF* (1.2 kb) and *aroG* (1.1kb), that is part of a single transcriptional unit. Bacterioferritin co-migratory protein (Bcp) has in several organisms been shown to play a role in oxidative stress resistance (20,54,147). It belongs to the thiol-specific antioxidant/alkyl hydroxyperoxidase family, detoxifying hydrogen peroxide in a similar way to the AhpC (64). The *bcp* defective mutant from *P. gingivalis* W83 designated FLL302, showed increased sensitivity to hydrogen peroxide at concentrations of 0.25mM when compared to the wild-type, was black pigmented like the parent strain and had similar rates of hemolysis, β hemolysis and UV sensitivity. This isogenic mutant also showed a 30% reduction in Arg-X and Lys-X activity during late exponential growth phase. There was also no detectable difference in the virulence potential of the *bcp* defective mutant when compared with the parent strain in the murine mouse model and demonstrated decreased aerotolerance when compared with the wild-type (67).

### RecA is Involved in DNA Repair in *P. gingivalis*

The *recA* of *P. gingivalis* is involved in DNA repair, and the deduced protein shows high similarity to the other Gram-negative anaerobes (42). The *recA* isogenic mutant (FLL32) is non black pigmented and has no detectable β-hemolysis on brucella blood agar plates, in contrast to wild-type W83 (parent strain), which is black pigmented

and hemolytic. Additionally, the *recA* mutant showed increased sensitivity to ultraviolet radiation and had reduced Arg-X and Lys-X specific proteolytic activities, though the transcription of these genes was unaffected. Monospecific, polyvalent antibodies against the gingipains identified them in the extracellular medium and suggest a difference in the processing of these proteases. This mutant was also significantly less virulent than the wild-type in the murine model and provided partial protection to animals against a subsequent lethal challenge by the wild-type strain. There was also increased autoaggregation of this mutant, suggesting changes in the surface membrane proteins (2). Using *in vitro* expression technology (IVET) with pFLL115 (suicide vector containing erythromycin/clindamycin resistance gene and the promotorless *xa-tetA* (Q) 2 cassette, it was demonstrated that the *recA* gene was expressed during infection of the murine host (89). The effect of environmental signals on *recA* promoter activity was examined using a transcriptional fusion of the *recA* promoter region with pFLL115. Exposure to agents that induce DNA damage did not elicit a change in reporter activity, which remained constant, suggesting that the *recA* is not controlled by an inducible SOS-like regulatory system (as observed in *E. coli*). Growth phase and pH changes had no effect on *recA* activity. In contrast, changes in temperature, calcium concentration and iron levels resulted in significant changes to the *recA* gene expression levels (90).

## Virulence Modulating Gene A

The *vimA* gene (virulence modulating gene A) is downstream of *recA* and codes for a unique 32 kDa protein. When this gene was inactivated by allelic exchange, the resulting phenotype of the stain (FLL92) was non black pigmented, has reduced Arg-X and LysX specific proteolytic activity, lacked beta hemolytic and hemagglutinating activities, showed increased autoaggregation and was nonvirulent when tested in the murine mouse model. The reduced gingipain activity was not associated with changes in

the transcription levels of the gingipain genes (1). This is further supported by the presence of the proenzyme form of the gingipains (105). There was also a late onset of gingipain activity in FLL92 which was mostly soluble, and had little or no cell-associated activity (105,143). The recombinant VimA can interact with the gingipains (Fig. 1.9.) in addition to several proteins, including HtrA, β-lactamase, sialidase and RegT, which are known in other bacterial systems to be involved in post-translational regulation Table 1.2. (144). There are 26 amino acid residue of VimA on HtrA and hypothetical protein PG1833, which may be a direct site of protein interaction. Sera from animals immunized with *P. gingivalis* were able to recognize VimA, suggesting that the immune system is exposed to VimA during the course of an infection (144).

### Virulence Modulating Gene E

The *vimE* gene is downstream of *vimA* and is expressed independently of *vimA.* The isogenic mutant of this gene (FLL93) resulted in a phenotype that showed reduced Arg-X and LysX specific proteolytic activities that unlike FLL92 was not affected by the phase of growth. A defect in gingipain biogenesis similar to FLL92 was supported by the presence of the mRNA transcript for the gingipain genes. A 64-kDa immunoreactive band similar to that observed in FLL92 was observed in FLL923, using antibodies against the RgpB proenzyme. Dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (DNS-EGR-CK), which irreversibly binds the Rgp active site of Arg-X proteases in wild-type *P. gingivalis* and FLL92 cells grown to stationary phase, did not label any protein similar in size to the expected 48-kDa fluorescent band in FLL93 (141).

### Virulence Modulating Gene F

The VimF protein coded for by the *vimF* gene is a putative glycosyltransferase. The phenotype of the isogenic mutant (FLL95) is similar to that of FLL93, i.e. has

reduced gingipain, hemagglutinating, and hemolytic activity. The gingipains unlike that observed in FLL92 are membrane and extracellular associated. The membrane forms are of high molecular weight; these represent the unprocessed or partially processed gingipains. Additionally, no immunoreactivity was observed with MAb 1B5 (monoclonal antibody specific for carbohydrate modifications of membrane associated Rgp and LPS). Several high molecular weight bands greater than 191-kDA were also observed in the membrane fraction of FLL95 and FLL93, and represent unprocessed or partially processed HagA species. This suggests that *vimF* may be necessary for proper processing of the hemmaglutinin protein. Fibronectin (plays an important role in cellular signal transduction by interacting with integrins) was cleaved at significantly lower levels when incubated with the *vimF and vimE,* when compared to W83, which completely degrades fibronectin. Significantly, the *vimA* defective mutant was unable to cleave fibronectin, and it has been suggested that the reason for this may be due to absence of Kgp and RgpA on the surface of the cell (142).

## The *recA* Locus and Adhesion

The effect of protease active extracellular protein preparations from *P. gingivalis* on the adhesion properties of human epidermoid carcinoma KB cells, demonstrated that extracellular protein preparations with gingipain activity induced cell rounding and detachment from each other and from the surface of the culture dish. The level of cell death was found to be correlated with the protease-dependent activities of the isogenic mutants tested (FLL32 and FLL33). Interestingly, the kinetics of N-cadherin cleavage and loss of cell adhesion was different in FLL32 and FLL33. Preparations from FLL32 (low gingipain activity) did not induce the cytotoxic effects observed for *P. gingivalis* FLL33 and W83. The levels of integrin $\beta$1 in FLL33 preparations remained unchanged in

the first 12 hours of exposure to *P. gingivalis* FLL33 preparations, however at 24 hours a decrease in the intensity of the integrin β1 was observed. There were also statistically significant moderate levels of apoptotic cell death during long-term incubation of KB cells with extracellular protein preparations from *P. gingivalis;* suggesting that gingipain induced cell death may contribute to tissue destruction typical of periodontal disease (19).

## Proteins that Interact With VimA

PG2096, designated *regT* (regulator of gingipain activity at elevated temperatures) was determined by *in silico* analysis to have 62% homology with the gene for Sgp G protein from *Streptococcus mutants*; additionally it had several RNA binding sites but no DNA binding sites. The isogenic *regT* mutant (FLL205) showed increased autolysis when compared to the parent strain when grown at 42°C up to 24 hours though there was no significant difference in the growth rate; however, when grown under oxidative stress, the *regT* defective mutant was more resistant to hydrogen peroxide than the wild-type. Gingipain activity of FLL205 was demonstrated to be dependent on the temperature at which it was grown. At 37°C, gingipain activity was lower than the wild-type, while at elevated temperature, gingipain activity was increased when compared to the wild-type. Though there was no change in the expression level of gingipain genes in (127) the wild-type compared with the mutant, immunoblot analysis showed that the catalytic domain of the Arg-X from cells grown at 42°C was more stable in the FLL205 than the wild-type. These results indicate that RegT facilitates the normal maturation/processing of gingipains at normal temperature at the posttranscriptional level (140)

HtrA in several organisms has been shown to be involved in protein folding and maturation in addition to degradation of misfolded proteins (58,68). Inactivation of this gene in *P. gingivalis* (FLL203) resulted in a phenotype that was similar to the wild-type, i.e. was black-pigmented and β-hemolytic. This mutant strain was more sensitive to oxidative stress when compared to the wild-type, and this sensitivity was most pronounced during the stationary growth phase. FLL203 also grew at a slower rate at 37°C, but grew more slowly at 42°C when compared to the wild-type; additionally, its growth did not exceed $OD_{600}$ of 0.9. There was no significant difference in the Arg-X activity between the mutant and wild-type strain at 37°C; however, after heat treatment, wild-type Arg-X activity increased by 7% while Arg-X activity decreased by 13% relative to an untreated control. No Arg-X immunoreactive bands were observed in the *htrA*-defective mutant. These results point to a role for HtrA in gingipain regulation at elevated temperatures (117).

**DNA Damage and Repair**

The repair activity of a mutagenic 8-oxoG lesion in *P. gingivalis* is dissimilar from *E. coli*, as enzymatic removal o f8-oxo-G occurs by DNA cleavage several bases away from the lesion. Greater 8-oxoG repair activity was observed in FLL92 compared with the wild-type. The cleavage pattern for 8-oxoG lesions was also demonstrated to be independent of the position of the 8-oxoG within the fragment (66,67). In comparison to other oral anaerobes, only *P. gingivalis* demonstrated a different pattern for the enzymatic removal of 8-oxoG, suggesting that this repair mechanism might be unique to *P. gingivalis.* Though our lab previously speculated that the repair mechanism for the 8-oxo-G lesion could a nucleotide excision repair like mechanism, further studies have indicated that this is not the case. The *uvrB* defective mutant from *P. gingivalis* was more

sensitive to UV irradiation that the wild-type strain and there was no difference in sensitivity to oxidative stress in the *uvrB*-defective mutant compared to the wild-type. Additionally, repair of oxidative stress-induced DNA damage was similar for both the wild-type and the *uvrB* defective mutant; this rules out *uvrB*-dependent nucleotide excision repair as a means of repairing 8-oxo-G lesions. Several lesion specific binding proteins were observed to bind the oligonucleotide carrying the 8-oxo-G, including PG10376. These proteins are suggested to form part of a unique mechanism for the removal and repair of 8-oxo-G lesions (52).

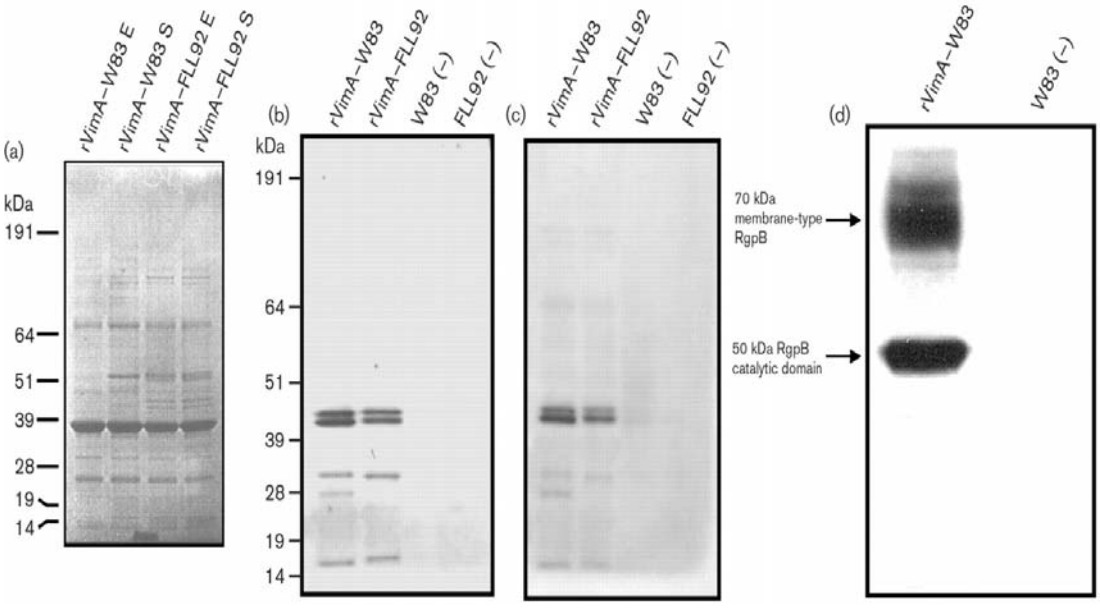


Fig. 1.9. rVimA interacts with gingipains RgpA, RgpB and Kgp. (a) Eluates from interaction studies with cell lysates of P. gingivalis W83 and FLL92 in the exponential phase (E) or stationary phase (S) were separated by SDS-PAGE and stained with SimplyBlue Safestain. (b–c) Eluates from protein interactions with lysates prepared from cells grown to late exponential phase were analysed by immunoblotting using gingipain-specific antibodies: anti-RgpA (b), anti-Kgp (c) or anti-RgpB (d). W83 (–), P. gingivalis W83 incubated with beads only; FLL92 (–), P. gingivalis FLL92 incubated with beads only (negative controls) (144).

Table 1.2. Proteins that interact with recombinant VimA

| Gene ID | Protein | Description |
|---|---|---|
| PG1101 | Alanyl-tRNA synthase | Translation |
| PG0535 | HtrA | Serine periplasmic protein |
| PG1833 | Conserved hypothetical protein | Unknown |
| PG1337 | Hypothetical protein | Unknown |
| PG0324 | Conserved hypothetical protein (putative sialidase) | Unknown |
| PG1768 | RgpA | Arg-gingipain |
| PG0461 | RgpB | Arg-gingipain |
| PG1605 | Kgp | Lys-gingipain |
| PG0010 | β-Lactamase | Energy/sugar metabolism |

## Elucidating the Role and Function of the VimA

We have previously shown that inactivation of the *vimA* gene which is part of the *bcp-recA-vimA-vimE-vimF-aroG* operon, alters glycosylation in *P. gingivalis* and plays an important role in protease activation in this organism. Furthermore, the gingipain RgpB proenzyme was secreted in the *vimA*-defective mutant, whereas both RgpA and Kgp was missing from the cell membrane (105,143). Protein-protein interaction studies using the 39 kDa recombinant VimA protein demonstrated interaction with the gingipains. In addition, other proteins known to be associated with carbohydrate metabolism and protease maturation also interacted with the rVimA protein (144). Collectively, these data suggest that VimA may be part of a protein complex that is involved in gingipain biogenesis/glycosylation, or may modulate other proteins that can directly/indirectly affect this process or cell surface structures. There is a gap in our understanding of the effects of VimA on cell surface structures; therefore, we will explore the hypothesis that the VimA of *Porphyromonas gingivalis* plays an important role in cell surface biogenesis. The following specific aims will be used to address the aforementioned hypothesis:

### Specific Aim One: Identification of Membrane and Extracellular Proteins Affected by the VimA Mutation

Our lab has previously shown that VimA is involved in several membrane related processes, including: gingipain maturation, LPS biosynthesis, and carbohydrate biosynthesis. SDS PAGE analysis of membrane and extracellular proteins of FLL92 versus the wild-type reveals a differential protein profile, this leads us to hypothesize that the *vimA* mutation is affecting the maturation and anchorage of several proteins. We will explore this hypothesis through mass spectrometric analysis and in silico analysis of membrane and extracellular proteins.

## Specific Aim 2: Functional Characterization of *vimA's* Involvement in Surface Biogenesis

The auto aggregation phenotype observed in the *vimA* mutant in addition to its differential carbohydrate profile points to a likely role in capsular formation, fimbrial expression and post translational modification of proteins. Using a combination of electron microscopy, lectin binding assays and bioinformatics, we aim to determine the involvement of VimA on surface related macro structures

## Specific Aim 3: Exploring VimA's Role as a Possible Acyl CoA N-acyl Transferase

VimA is predicted by *In silico* analysis to belong to the Acyl-CoA N-acyl transferase; additionally, its predicted tertiary structure is similar to the FemXAB family of proteins which have been demonstrated to be involved in peptidoglycan formation. In this specific aim we will elucidate the possible role of *vimA* as a putative acyl transferase by $^{3}H$ labeled palmitic acid assays, antibiotic sensitivity assays, isolation and characterization of the peptidoglycan and determination of chemical structure variability.

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CHAPTER TWO

# THE ROLE OF VimA IN CELL SURFACE BIOGENESIS IN *Porphyromonas gingivalis*

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Running title: The role of *vimA* in cell surface biogenesis

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Microbiology (2010), 156, 2180-2193

**Summary**

The *Porphyromonas gingivalis vimA* gene has been previously shown to play a significant role in the biogenesis of gingipains. Further, in *P. gingivalis* FLL92, a *vimA* defective mutant, there was increased auto-aggregation, suggesting alteration in membrane surface proteins. In order to determine the role of the VimA protein in cell surface biogenesis, the surface morphology of *P. gingivalis* FLL92 was further characterized. Transmission electron microscopy demonstrated abundant fimbrial appendages and a less well defined and irregular capsule in FLL92 compared to the wild-type. In addition, Atomic Force microscopy showed a smoother surface for the wild-type strain in contrast to the FLL92. Western blot analysis using anti-FimA antibodies showed a 41 kDa immunoreactive protein band in *P. gingivalis* FLL92 which was missing in the wild-type *P. gingivalis* W83 strain. There was increased sensitivity to Globomycin and Vancomycin in FLL92 compared to the wild-type. Outer membrane fractions from FLL92 had a modified lectin binding profile. Furthermore, in contrast to the wild-type strain, 9 proteins were missing from the outer membrane fraction of FLL92, while 20 proteins present in that fraction from FLL92 were missing in the W83 strain. Taken together, these results suggest that the VimA protein affects capsular synthesis, fimbrial phenotypic expression and plays a role in the glycosylation and anchorage of several surface proteins.

## Introduction

A variety of bacterial cell surface components including: capsule, polysaccharides, proteases (gingipains), hemmaglutinin, lipopolysacharides, major outer membrane proteins and fimbriae contribute to the virulence of *Porphyromonas gingivalis* [reviewed in (35,85)]. This organism, a black-pigmented, Gram-negative anaerobe, is an important etiologic agent of periodontal disease and is associated with other systemic illnesses including cardiovascular disease (10,12,17,47). Cell surface components like the fimbriae (3,45,83), lectin-like adhesins (9) and gingipains (42,57,60) are integrally involved in the adhesion, invasion and colonization of periodontal tissue. In addition, the gingipains are involved in several cellular processes (33) including: growth, inactivation of cytokines and their receptors, platelet aggregation, attenuation of neutrophil antibacterial activities, increasing vascular permeability and apoptosis of gingival epithelial cells (73). Gingipains consist of a Lysine specific protease (Kgp) and an Arginine specific protease (Rgp) – which is further subdivided into RgpA and RgpB; these cleave proteins after arginine residues, and are encoded by the rgpA and rgpB genes respectively. The mature form of RgpA contains a catalytic domain and a hemagglutin domain while RgpB possesses only a catalytic domain. Lysine specific protease (KGP) (28,44,58) – cleave proteins after lysine residues.

Post-translational modifications of surface components like the gingipains, fimbriae and LPS play a key functional role in regulating the virulence of the organism. The maturation pathway of the gingipains are linked to the biosynthesis of surface carbohydrates (56,70) and several other proteins including the PorR (70), PorT (49,67), Sov (67) and VimA (77,79,80). Arg-X also play a role in processing fimbriae protein precursors serving as a proteinase for fimbrillin maturation (30,48). The LPS of *P. gingivalis* which has been recently shown to contain two macromolecules: O-LPS (O antigen attached to a lipid A core) and A-LPS (phosphorylated branched mannan

repeating unit attached to a lipid A core), undergo several stages of assembly including catalysis by a relaxed-specificity ligase which is able to attach either a branched phosphorylated mannan or a tetrasaccharide repeating unit to the Lipid A core (63). Lipid A is also present in several structural forms (32) and is likely a function of strain differences as well as environmental cues.

The *P. gingivalis vimA* gene which is part of the recA operon and codes for a 39 kDa protein (putative Acyl CoA N-acyltransferase) has been previously shown to be part of the maturation pathway for gingipains (1,80). Though the mechanism for gingipain regulation has not been thoroughly elucidated, we have demonstrated that *vimA* modulates the phenotypic expression of the gingipains. Additionally, inactivation of this gene resulted in a non-black pigmented strain designated *P. gingivalis* FLL92, which showed reduced levels of proteolytic, hemagglutinating and hemolytic activities (1,53). Using a mouse model, the virulence of *P. gingivalis* FLL92 was shown to be drastically reduced when compared to wild- type W83 strain. The reduced proteolytic activity observed in this *vimA*-defective mutant is as a result of a defect in the post-translational regulation of these genes. This is further supported by the presence of the proenzyme form of the gingipains in addition to their unaltered transcription in the *vimA*-defective mutant (53). There is also a late onset of gingipain activity in FLL92 which is mostly soluble with little or no cell-associated activity (53,79). Other studies have shown that the recombinant VimA can interact with the gingipains in addition to several proteins, including HtrA, β-lactamase, and sialidase which are known in other bacterial systems to be involved in post-translational regulation (80). It still remains unclear whether the *vimA* gene product is part of a central mechanism that may be involved in the maturation or post-translational regulation of other cell surface proteins in *P. gingivalis*.

In the present study, we further characterized the cell surface of the *vimA*-defective mutant of *P. gingivalis.* We investigated the role of VimA on capsule and

fimbriae formation and outer membrane protein expression. In addition, the effect of cell surface changes on antibiotic resistance was also evaluated.

## Material and Methods

### Bacterial Strains and Growth Conditions

*P. gingivalis* strains (W83, ATCC 33277 and FLL92) were grown in either Brain Heart Infusion (BHI) broth (Difco Laboratories) supplemented with cysteine (0.1%), vitamin K (0.5 µg/ml) and hemin (5 µg/ml) or in Trypticase Soy Broth containing menadione and hemin (TSBKH). Solid medium was prepared by supplementation with 1.5% agar and 5% defibrinated sheep blood (Hemostat laboratories). All cultures were incubated at 37˚C in an anaerobic chamber (Coy Manufacturing) in 80%$N_2$, 10% $H_2$ and 10% $CO_2$. Growth rates were determined spectrophotometrically at 600nm (optical density).

### Biofilm Formation Assay

Biofilm formation was assayed in 96-well microtiter dishes (Lamont personal communication). Briefly, *P. gingivalis* was cultured overnight in TSBKH media. One ml of the overnight culture (mid-log phase) was centrifuged at 11,000 rpm for 2 minutes at room temperature. The pellet was collected and washed twice with 1 ml of PBS, then resuspended in a 1 ml mixture of TSBKH and PBS (1:2). Aliquots of the sample were added to each well. Plates were then incubated with a slight rotation for 24 hours in an anaerobic chamber, to allow for the development of biofilm. After removal of the culture supernatant, the plates were washed twice by immersion in distilled water then allowed to air dry for 1 hour. The biofilm was stained with 0.5% safranin for 15 min (100 µl per well), then washed twice with distilled water. Ethanol (95%) was added to solubilize the

safranin which was transferred to a new microtiter dish. Biofilm formation was obtained by determining the absorbance with a plate reader at 490 nm (11).

## Analysis of *P. gingivalis* Strains by RT-PCR

Total RNA was extracted from *P. gingivalis* at the early (O.D. 1.0) phase of the growth cycle, using the SV Total RNA Isolation System (Promega). RT-PCR was performed using primers specific for the FimA (Fwd – GGCAFAACCCGTTGTAGAAA, Rve – GACCAAAGAATTGCCGAAAA) and 16S ribosomal gene product (6). The protocol was done according to the one step RT-PCR kit (Invitrogen) with 1µg of template RNA in 50µl of RT-PCR mixture. RT-PCR in the absence of reverse transcriptase served as the negative control.

## Protein Electrophoresis and Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the manufacturers instructions (Invitrogen) on 10% bis-tris separating gel in MOPS (morpholinepropanesulfonic acid) SDS running buffer. Proteins on SDS-PAGE gels were electrophoretically transferred to a nitrocellulose membrane (Whatman Optitran BA-S 85) and the blotted membranes immunostained with polyclonal anti-FimA (36). Lectin studies using Agaricus bisporus [ABA; specific for Galactose (β 1,3) N-Acetylgalactosamine], Concanavalin A [ConA; specific for α- mannose & α-glucose], Dolichos biflorus [DBA; specific for N-acetyl-α-D-galactosamine], Erythrina cristagalli [ECA; specific for Galactose (β 1,4) N-Acetylglucosamine], Glycine Max [SBA; specific for N-acetyl-D-galactosamine], Limulus polyphemus [LPA; specific for Sialic Acid (N-Acetyl neuramic acid)] and Triticum vulgaris [WGA; specific for N-acetyl-β-D-glucosamine], were done according to the recommendations of the manufacturer (Sigma

Aldrich). In brief, blots were blocked in phosphate buffered saline (PBS) containing 2% TWEEN for 2 minutes at 20˚C. Blots were then rinsed twice in PBS, and incubated with 1 µg/ml of lectin-peroxidase in PBS containing 0.05% TWEEN, with 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ for 16 hours at 20˚C. Surplus lectins were removed by rinsing with PBS; the blots were developed using the DAB staining kit with nickel enhancement (Vector Laboratories) (37).

## Microscopic Examination

Atomic Force Microscopy (AFM) and transmission Electron microscopy (TEM) were used to provide detailed visuals of the surface morphology. AFM was done using W83 and FLL92 stains grown overnight to OD 1.2, washed twice in 0.1M PBS buffer pH 7.4 and mounted on a cover slide. AFM was performed using the Innova Scanning Probe Microscope (Veeco Instruments Inc) in air using the tapping mode. Images were captured in the height mode with a resolution of 256x256 (62). Transmission Electron Microscopy was performed using the Philips Tecnai 12 TEM as per the method of Hyatt (27). Briefly, Formvar-carbon coated grids were prepared; the Formvar support was removed by placing the grids in an atmosphere of solvent vapor. Grids were then placed on a wire mesh in a glass Petri dish with carbon tetrachloride below the wire mesh. One µl of the serially diluted sample was placed under the carbon side of a 4x5 mm square of mica (approximately twice the size of an EM grid). The grid was washed in 0.5% acetic acid then acetone. The carbon film was broken to free the specimen grid, after which the grid was placed in stain solution - neutral 1% aqueous phosphotungstic acid for 30 seconds. After blotting dry, the grid was examined using the Tecnai TEM (FEI).

## Localization of FimA in *P. gingivalis* by Immunogold Straining

Anti-FimA antibodies (36) were purified and used at a working concentration of 1:100. The colloidal gold conjugate, 10 nm in size (Aurion), was diluted using incubation buffer (20mM phosphate buffer, 150 mM NaCl pH7.4, 0.2% BSA, 15mM $NaN_3$) to 1:1000. A checker board titration of primary antibody to immunogold conjugate was made to identify the optimal working concentration. Processed nickel grids were subjected to charging of the processed *P. gingivalis* strains for a period of 1 hour, then washed 20 times in 0.025M TRIS buffer and lightly blotted. Grids were subsequently blocked with 5% Bovine serum albumin in 0.025M TRIS buffer for 15 minutes at room temperature, then incubated in diluted antibody-gold complex for 4 hours at 37$^{o}$C. Staining was done using uranyl acetate (0.8g of uranyl acetate dissolved in 10ml of absolute ethanol) and freshly prepared lead citrate (10-40mg lead citrate, 10ml of filtered water, 100µl of 10N NaOH). Grids were immersed in uranyl acetate for 7 minutes at room temperature and then washed, once by immersion in 25% ethanol and twice by immersion in water. Grids were dried on filter paper for 10 minutes at room temperature, and then floated on lead citrate drops for 5 minutes. Following two washes by immersion in 0.02N sodium hydroxide and drying, grids were visualized using the Philips Tecnai 12 TEM.

## Hydrophobicity Assay

Hydrophobicity assays of *P. gingivalis* strains were based on adhesion to hexadecane according to the method of Grivet (22). Bacteria were harvested during exponential growth phase by centrifugation at 4˚C for 15 minutes then washed with 0.1M PBS (pH 7) and resuspended in PBS. The optical density of the samples was adjusted to 0.85 at 600 nm. Three ml of bacterial suspension was placed in a polystyrene tube to which 400 µl of hexadecane was added. No hexadecane was added to the control

suspensions. Suspensions were equilibrated in a water bath at 37˚C, vortexed for two 30 second periods with five second intervals and allowed to stand until the phases separated. The lower aqueous phase was carefully removed and its optical density determined at 600 nm. The values obtained were expressed as the percentage of bacteria remaining in the aqueous phase compared with the control suspension.

## Determination of Minimum Inhibitory Concentrations (MIC)

Serial dilutions of antibiotics were used to determine MIC values in broth. These experiments were done in duplicates. In brief, broth macro dilutions of W83 and FLL92 were carried out using tubes containing 9 ml BHI-HK broth plus 1 ml of antibiotic dilution. Tubes were examined for turbidity after incubation at 37ºC for 72 hours in an anaerobic chamber (84). The MIC was taken as the concentration at which no visible growth was observed.

## Labeling of *P. gingivalis* with [$^{3}$H]-Palmitic Acid

*P. gingivalis* cells were labeled according the protocol of Shoji (69). Briefly, 5 ml cultures of W83 and FLL92 were incubated for 24 hours in BHI-HK in the presence of 50 µCi/ml of Palmitic acid [9,10(n)-$^{3}$H] (MP Biomedicals). Cells were then centrifuged, washed in PBS buffer and homogenized using glass beads to derive total proteins. Protein fractions were subsequently run on an SDS-PAGE gel. Radiolabeled proteins were detected using autoradiography as previously reported (82).

## Bioinformatics Analysis of VimA

Protein modeling of Vim A was conducted using I-Tasser (87,88). The subcellular location of the protein was predicted from its amino acid sequence using SignalP 3.0

(50), Phobius (29) and PsortB (19). Protein motifs and domains of VimA were identified using PPSearch (59) and Superfamily (20).

## Cell Fractionation

The Sarkosyl method was used to prepare outer membrane fractions of *P. gingivalis* (61). In brief, *P. gingivalis* cells were harvested from a 500 ml culture during the exponential and stationary phase of the growth cycle (OD 0.6-1.0 and 1.5-1.8 respectively). Cells were pelleted at 9000xg for 30 minutes at 4ºC then resuspended in 5 ml of 20 mM Tris HCL (pH 7.4), 10 mM EDTA, 10 mM of Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and 1% sodium lauryl sarcosinate. Cells were disrupted by passage through a French pressure cell at 109 MPa. Whole cells were removed by further centrifugation at 5000xg for 20 minutes at 4ºC. The outer membrane fraction was separated by centrifugation (100,000xg for 30 minutes), and then the pellet was washed three times in 0.5% sodium lauryl sarcosinate and resuspended in 20 mM Tris HCL (pH 7.4). Extracellular fractions were prepared from cell-free culture fluid precipitated with 60% acetone (-20ºC) (80). The protein pellet was resuspended in 7 ml 100 mM Tris-HCL buffer (pH 7.4) in the presence of 1 mM N-p-tosyk-L-lysine chloromethyl ketone (TLCK), dialyzed for 48 hours against the same buffer then stored at -20ºC. Total proteins were harvested from a 50 ml culture during the late exponential phase. Cells were pelleted at 8000xg for 30 minutes then resuspended in 1 ml of 25 mM Tris HCL (pH 8.0) with a protease inhibitor cocktail tablet (Complete EDTA-free, Roche Indianapolis, IN), RNase and DNase. Resuspended cells were then transferred to a 2 ml cryogenic tube containing 1 g of 0.1 mm glass beads. Cells were lysed in a Beadbeater homogenizer for 4 minutes with 30 second intervals with 1 minute cooling on ice. Samples were then centrifuged (10,000xg) for 12 minutes 4ºC. The upper aqueous layer containing the total protein fraction was removed and stored on ice or at -20ºC.

## Digestion of *P. gingivalis* Wild-type and FLL92 Proteins

Late exponential and stationary membrane proteins were run on a 10% bis-Tris gel (Invitrogen, Carlsbad, CA) in 1X MOPS running buffer for 1.5 cm, then visualized by staining with SimplyBlue safe stain (Invitrogen) (25). After destaining in water, the gel was cut into 1-2 mm slices. Gel slices were subsequently dehydrated in acetonitrile and dried in a vacuum centrifuge for 30 minutes. The gel slices were incubated for 1 hour at 60ºC in a solution containing 20 µl of 20mM Dithiothreitol in 100mM $NH_4HCO_3$ (enough to cover the gel pieces). The Dithiothreitol solution was replaced with an alkylating solution (20 µl of 200 mM iodoacetamide in 100 mM $NH_4HCO_3$) after cooling the proteins to room temperature. Gel slices were further incubated at ambient temperature for 30 minutes in the dark, followed by two washes with 150 µl of 100 mM $NH_4HCO_3$, then finely minced with a flame sealed polypropylene pipette tip, dehydrated by the addition of acetonitrile, and vacuum dried. Following an overnight incubation of the gel pieces with 20 µl digestion buffer [1 µl of mass spectrometry (MS)-grade trypsin (www.promega.com) in 50 mM acetic acid with 1 µl of 100 mM $NH_4HCO_3$], the digestion reaction was stopped with 10 µl of 5% formic acid. After transferring the digest solution (aqueous extraction) to a 0.65 ml siliconized tube, 30 µl of 50% acetonitrile with 0.1% formic acid was added, the mixture was vortexed for 3 minutes, centrifuged and then sonicated for 5 minutes. The process was repeated and both extractions pooled and concentrated to 10 µl in a vacuum centrifuge. Peptide extraction was accomplished using standard $C_{18}$ ZipTip technology following the manufacturer's directions (Millipore, Bedford, MA).

## MS and Data Analysis

An LCQ Deca XP Plus system (www.thermo.com) with nano-electrospray technology (www.newobjective.com) consisting of a reverse phase $C_{18}$ separation of

peptides on a 10 cm by 75µm capillary column using Microm Magic RP-18AQ resin (www.michrom.com) with direct electrospray injection to analyze the extracted peptides from each gel piece (25). A four part protocol was used for the MS and MS/MS analyses, this included one full MS analysis (from 450 to 1750 m/z) followed by three MS/MS events using data-dependent acquisition, where the most intense ion from a given full MS scan was subjected to collision-induced dissociation, followed by the second and third most intense ions. The nanoflow buffer gradient was extended over 45 minutes in conjunction with the cycle repeating itself every 2 seconds, using a 0-60% acetonitrile gradient from buffer B (95% acetonitrile with 0.1% formic acid) developed against buffer A (2% acetonitrile with 0.1% formic acid) at a flow rate of 250 to 300 nl/min, with a final 5 minute 80% bump of buffer B before re-equilibration. In order to move the 20 µl sample from the autosampler to the nanospray unit, flow stream splitting (1:1000) and a Scivex 10 port automated valve (Upchurch Scientific, Oak Harbor, WA) together with a Michrom nanotrap column was used. The spray voltage and current were set at 2.2kV and 5.0µA, with a capillary voltage of 25V in positive ion mode. 160°C was used as the spray temperature for peptides. Data collection was achieved using the Xcalibur software (Thermo Electron), then screened with Bioworks 3.1. MASCOT software (www.matrixscience.com) was used for each analysis to produce unfiltered data and out files. Statistical validation of peptide and protein findings was achieved using X! TANDEM (www.thegmp.org) and SCAFFOLD 2 meta analysis software (www.proteomesoftware.com). The presence of two different peptides at a probability of at least 95% was required for consideration as being positively identified. Confirmation of individual peptide matches was achieved using the BLAST database (www.oralgen.lanl.gov)

## Results

### In silico Analysis of VimA Reveals Close Similarity to the Fem Family of Proteins

Using I-TASSER the putative structure of the VimA was modeled, this structure has a confidence score of 0.54 (Fig. 2.1). Several binding sites were also predicted (Fig. 2.2) the predicted structure is similar to the FemA of *Staphylococcus aureus* (TM-score 0.81) and FemX of *Weisella viridiscens* (TM-score 0.89). The gene ontology is predicted to include a role in one or more of the following: peptidoglycan biosynthetic process, cellular component biogenesis, cell wall biogenesis, peptidoglycan based cell wall biogenesis, metabolic process, biosynthetic process, carbohydrate biosynthetic process, primary metabolic process, carbohydrate metabolic process and peptidoglycan metabolic process. SignalP 3.0 and PSortb predicted no signal peptide in the VimA sequence, suggesting that this protein is not secreted. Phobius scores the likelihood of VimA being a non-cytoplasmic protein at 0.83. Using EMBL protein interaction software v.8.1, predicts VimA to directly interact with several proteins including several uncharacterized putative proteins of unknown functional etiology. The cysteine proteases (PG0506-RgpB) are associated with VimA with a score of 0.808. The bacterioferritin combinatory protein (BCP) has an interaction score of 0.582 and is involved in storage of Iron in the form of hydrated ferric oxide. Lys1 mediates lysine biosynthesis through the enzyme saccharopine dehydrogenase, while RecA has multiple activities, all related to DNA repair. Position specific iterative protein classifier and Superfam 3.0 predicts VimA to belong to the DUF482/CH1444 and/or Acyl CoA N-acyltransferase superfamily.

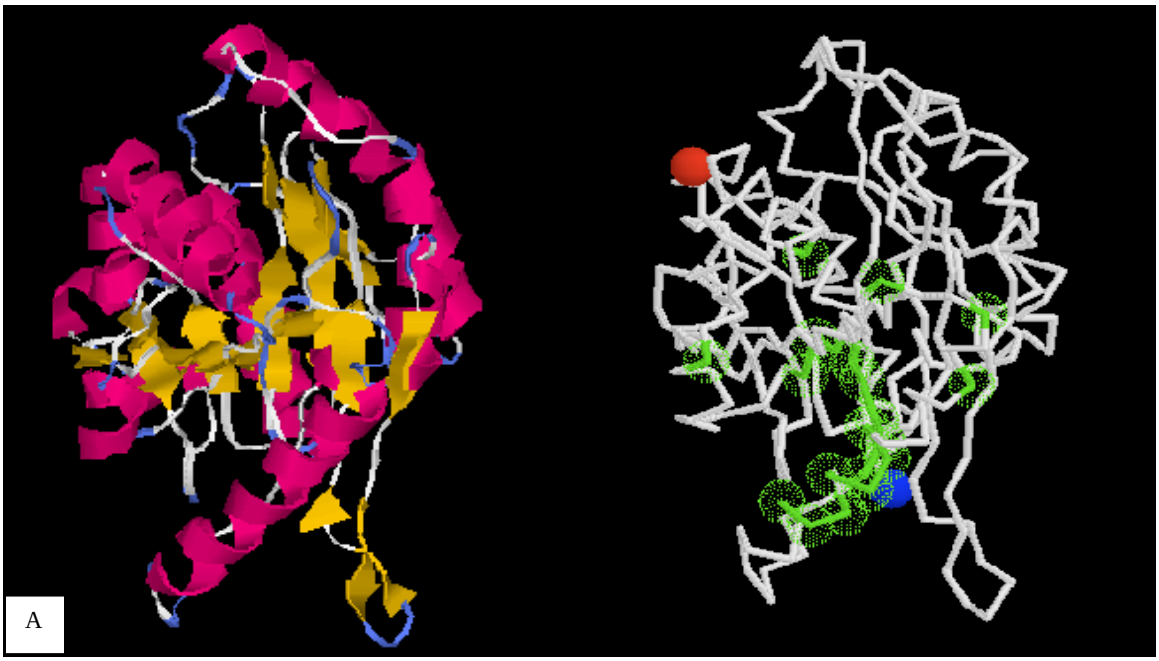


**Fig. 2.1.** (A) Model of the VimA protein (c score 0.54). (B) Predicted binding site residues (shown in green): Leu:55 GLY:56 SER:57, PHE:71 ARG:72 ALA:73, VAL:77 HIS:78 MET:84, TYR:95 SER:96 LYS:97, TYR:58 SER:59, ARG:75 ARG:76, HIS:88 PHE:91, and GLN:108 TRP:297

## The Surface Morphology is Affected by the *vimA* Mutation

Auto-aggregation in *P. gingivalis* seems to be correlated to the absence of a capsule and the presence of fimbriae (11). Atomic force and transmission electron microscopy were used to visualize likely differences between the mutant and the wild-type. Micrographs of W83 (Fig. 2.2. A) and FLL92 (Fig. 2.2. B) revealed differences in the capsular structure. The Capsule of W83 was solid and well defined in contrast to the capsule of FLL92 which was less defined and irregular. AFM micrographs of W83 (Fig. 2.2. C) and FLL92 (Fig 2.2. D) showed FLL92 to have distinct fine structures, resulting in a corrugated appearance, in contrast to the smooth morphology of W83. FLL92 cells also tended to clump together. Immunogold staining of W83 (Fig. 2.2. E) and FLL92 (Fig. 2.2. F) showed adherence of gold particles to abundant fimbrial appendages in FLL92 in contrast to few gold particles adhering in W83.

## Expression of Fimbrial Genes in the *vimA* Defective Mutant

The autoaggregation observed in FLL92 could have been as a result of changes to the fimbrial protein. It is also possible that VimA may also be involved in the post translational regulation of fimbrial expression. To confirm the presence of the mRNA transcript for the FimA gene, total RNA was isolated from wild-type and FLL92 strains grown to stationary phase. Using reverse transcriptase with specific oligonucleotide primers for an intragenic region of the *fimA* gene, a predicted 0.6 kb fragment was amplified for both the wild-type and FLL92 isogenic strain. Immunoblotting of outer membrane and total protein fractions of FLL92 and W83 with the FimA antibody revealed strong immunoreactivity with a 41-43 kDa band in both fractions of FLL92 (Fig. 2.3.). This band which corresponds with the expected size of FimA was absent in both fractions of W83.

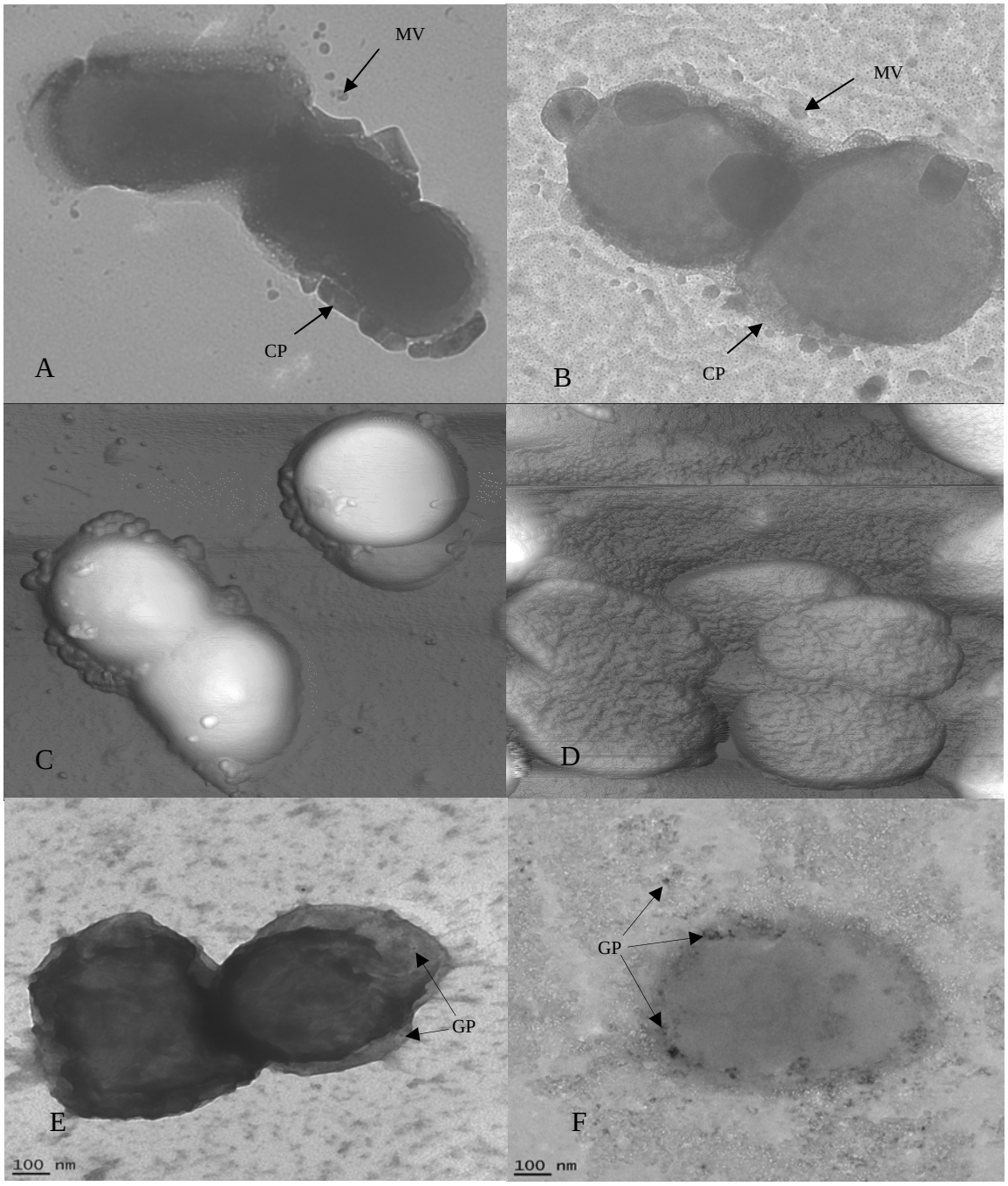


Fig. 2.2 TEM micrographs of W83 (A) and FLL92 (B) showing capsule (CP) with vesicles (MV). AFM micrographs of W83 (C) and FLL92 (D). Immunogold localization of *P. gingivalis* FimA in W83 (E) and FLL92 (F), visualized by TEM after incubation with anti FimA serum conjugated to 10-nm gold particles (GP).

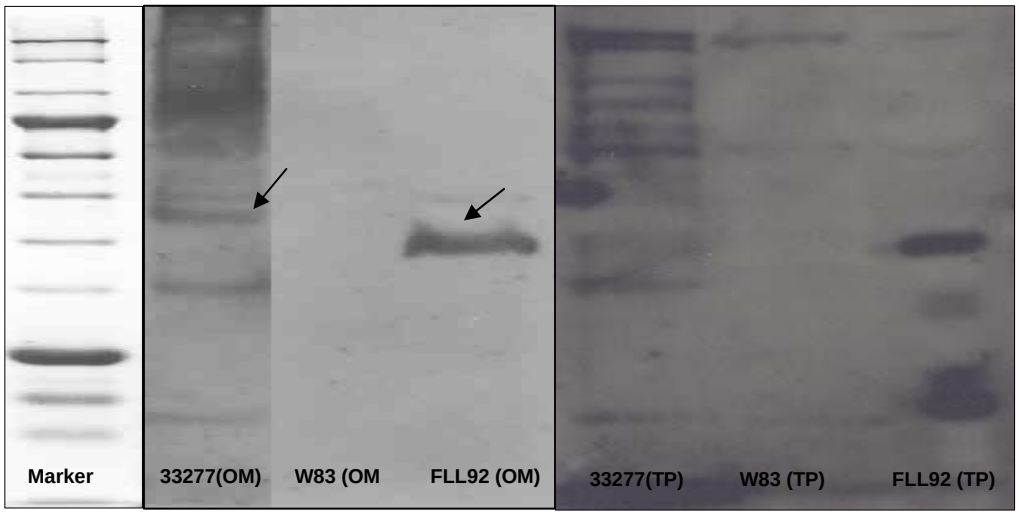


Fig. 2.3. Immunoblot analysis using anti-FimA antibody with outer membrane (OM) and total protein (TP) fractions of *P. gingivalis* ATCC 33277, W83 and FLL92. A 41kDa protein corresponding with the expected size of the FimA was observed in the *vimA* mutant.

## There is no Change in the Hydrophobicity or Ability to Form Biofilm in FLL92

Adhesion to hexadecane was used to determine the hydrophobicity of W83 and FLL92; ATCC 33277 served as a control (known to form biofilm and auto-aggregates). There was no change in the hydrophobicity profile of FLL92 compared to W83. 55% of ATCC 33277 cells adhered to hexadecane, this is reflective of their hydrophilic surface properties. The ability of the *vimA* defective mutant FLL92 to form biofilm as quantified by adherence to the surface of 96 well plates was unchanged when compared to the W83 (data not shown).

## Bacterial Cell Surface/Membrane Modifications Can Alter Antimicrobial Sensitivity

Several antibiotics with varying targets were employed in antibiotic sensitivity testing of FLL92. FLL92 showed increased sensitivity to Globomycin (10 µg/ml compared with 25 µg/ml in W83) and Vancomycin (5µg/ml compared with 10 µg/ml in W83)

## The VimA Affects Outer Membrane Proteins Glycosylated with Specific Carbohydrate Moieties

Previous reports from our laboratory have documented the changes in the glycosylation profile of FLL92 compared to wild-type W83 (79). To further confirm the role of VimA in post translational glycosylation of outer membrane proteins, lectins were used. Differential lectin bound protein profiles were observed using ConA, DBA, ECA, LPA and SBA (Fig. 2.4.). No discernable differences were observed using WGA or ABA.

## VimA Affects the Anchorage of Several Membrane Bound Proteins

Mass Spectrometric analysis of membrane fractions of W83 and FLL92 identified 20 proteins that were absent from the wild-type membrane fraction but present in the FLL92 fraction (Table 2.1.). While 13 of these proteins are hypothetical, PG0083, PG0581, PG0602, PG0703 and PG1496 are predicted to be involved in transport (http://www.oralgen.lanl.gov/). Nine proteins were identified as missing from the FLL92 fraction in contrast to the wild-type (Table 2.2.). These included three proteases: RgpA (PG1768), Kgp (PG1605) and Carboxypeptidase D (PG0212) in addition to PG0554 and PG1136 which are predicted to be involved in amino acid biosynthesis (http://www.oralgen.lanl.gov/). PG1857 which is predicted for involvement in glycolysis or gluconeogenesis (http://www.oralgen.lanl.gov/) was also identified as missing. Twenty one membrane proteins were identified as having variable spectral counts (Table 2.3.), corresponding with a fold change of 1.4 or greater. Ten of these proteins showed a positive fold change in the VimA mutant while 11 showed a negative fold change. The majority of these proteins were predicted to be involved in cell envelope biogenesis or transport (http://www.oralgen.lanl.gov/).

## Analysis of Missing Membrane Proteins Reveals Several Common Motifs

Using PPSearch (http://www.ebi.ac.uk/Tools/ppsearch/), several common domains were identified in the missing proteins identified by MS analysis (Table 2.4.). In PG1768 (RgpA), PG1605 (Kgp), PG0212 (Carboxypeptidase D) and PG0981 (Hypothetical) the most abundant motifs were myristoylation sites (13, 11, 49 & 37 respectively), Casein Kinase II phosphorylation motifs (19, 14, 29 & 35 respectively) and Protein Kinase C motifs (15, 16, 27 & 20 respectively).

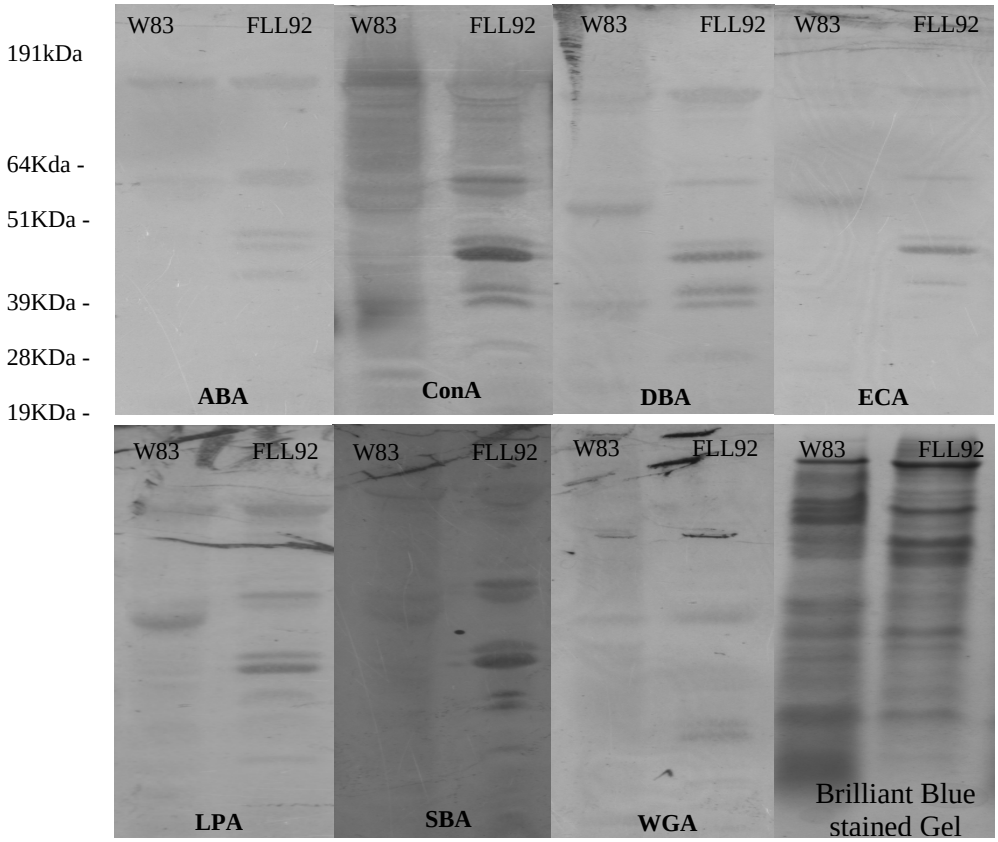


Fig. 2.4. Outer Membrane preparations from *P. gingivalis* W83 and FLL92 were transferred to nitrocellulose, blocked in PBS then incubated with lectin-peroxidase overnight. Peroxidase activity was detected using the DAB peroxidase substrate kit. Differential lectin binding was observed using ConA, DBA, ECA, LPA and SBA.

Table 2.1. MS analysis of aberrantly expressed proteins in FLL92

| Identified Proteins | Accession # | Putative Role/Function | M.W. | W83 S.C.V | FLL92 S.C.V |
|---|---|---|---|---|---|
| conserved hypothetical protein (possible alkaline secretion protease) | PG0083 | Transport and binding | 56 kDa | 0 | 4 |
| conserved hypothetical protein; possible lipoprotein | PG0219 | Unknown | 32 kDa | 0 | 82 |
| hypothetical protein | PG0302 | Unknown | 47 kDa | 0 | 7 |
| conserved hypothetical protein | PG0332 | Unknown | 22 kDa | 0 | 1 |
| hypothetical protein | PG0334 | Unknown | 82 kDa | 0 | 2 |
| conserved hypothetical protein | PG0373 | Unknown | 30 kDa | 0 | 16 |
| conserved hypothetical protein | PG0410 | Unknown | 48 kDa | 0 | 4 |
| periplasmic serine protease | PG0535 | Proteolysis | 53 kDa | 0 | 1 |
| tonB receptor tlr (C-terminal part) | PG0581 | Transport | 44 kDa | 0 | 4 |
| heme-binding protein/peripheral outer membrane chelatase | PG0602 | Transport and binding | 33 kDa | 0 | 4 |
| probable biopolymer transport (tolQ) protein | PG0703 | Transport | 29 kDa | 0 | 3 |
| hypothetical protein | PG0809 | Unknown | 16 kDa | 0 | 2 |
| conserved hypothetical protein | PG0922 | Unknown | 55 kDa | 0 | 7 |
| probable outer membrane lipoprotein | PG1177 | Cell wall biogenesis | 21 kDa | 0 | 18 |
| conserved hypothetical protein | PG1308 | Unknown | 100 kDa | 0 | 24 |
| conserved hypothetical protein | PG1496 | Transport | 99 kDa | 0 | 19 |
| conserved hypothetical protein | PG1772 | Proteolysis | 98 kDa | 0 | 4 |
| hypothetical protein | PG1790 | Unknown | 10 kDa | 0 | 1 |
| outer membrane protein (immunogenic 23 kDa lipoprotein) | PG1793 | Cell wall biogenesis | 23 kDa | 0 | 44 |
| probable integral outer membrane protein | PG1840 | Cell wall biogenesis | 24 kDa | 0 | 24 |

Table 2.2. MS analysis of missing proteins in FLL92

| Identified Proteins | Accession # | Putative Role/Involvement | M.W. | W83 S.C.V | FLL92 S.C.V |
|---|---|---|---|---|---|
| carboxypeptidase D / immunoreactive 92 kDa antigen | PG0212 | Proteolysis | 92 kDa | 3 | 0 |
| hypothetical protein | PG0554 | Cell redox homeostasis | 38 kDa | 95 | 0 |
| conserved hypothetical protein | PG0981 | Unknown | 107 kDa | 3 | 0 |
| branched-chain amino acid aminotransferase | PG1136 | Amino acid biosynthesis | 38 kDa | 11 | 0 |
| peptidylarginine deiminase | PG1249 | Arginine & Proline metabolism | 62 kDa | 100 | 0 |
| porphypain polyprotein; lys-Xproteinase/hemagglutinin | PG1605 | Proteolysis/Pathogenesis/DNA replication & repair | 188 kDa | 60 | 0 |
| hypothetical protein | PG1722.1 | Unknown | 24 kDa | 5 | 0 |
| arginine-specific cysteine proteinase; gingipain | PG1768 | Proteolysis/Pathogenesis/DNA replication & repair | 186 kDa | 92 | 0 |
| glyceraldehyde 3-phosphate dehydrogenase | PG1857 | Glycolysis/gluconeogenesis | 36 kDa | 60 | 0 |

Table 2.3. MS analysis of proteins with different spectral count values in W83 and FLL92

| Identified Proteins | Accession # | Putative Role/Involvement | M.W. | Fold Change | W83 S.C.V | FLL92 S.C.V |
|---|---|---|---|---|---|---|
| probable outer membrane lipoprotein | PG0916 | Cell envelope | 63 kDa | 7.1 | 26 | 185 |
| possible outer membrane-associated protein | PG1893 | Cell envelope | 54 kDa | 4 | 40 | 10 |
| tonB-linked outer membrane receptor | PG0601 | Transport & Binding | 85 kDa | 3.6 | 5 | 18 |
| possible outer membrane-associated protein | PG1424 | Cell envelope | 61 kDa | 3.2 | 17 | 54 |
| outer membrane protein | PG0626 | Cell envelope | 42 kDa | 2.6 | 156 | 408 |
| possible lipoprotein | PG1600 | Unknown | 51 kDa | 2.2 | 5 | 11 |
| outer membrane protein | PG0627 | Cell envelope | 43 kDa | 2 | 129 | 256 |
| probable integral outer membrane protein | PG1592 | Cell envelope | 24 kDa | 1.9 | 29 | 56 |
| probable integral outer membrane protein | PG1562 | Cell envelope | 32 kDa | 1.6 | 11 | 17 |
| tonB-dependent outer membrane receptor | PG0170 | Transport | 112 kDa | 1.5 | 452 | 674 |
| receptor antigen B | PG0171 | Cell envelope | 56 kDa | -1.4 | 455 | 316 |
| tonB-linked receptor Tlr | PG0582 | Transport | 51 kDa | -1.4 | 14 | 10 |
| tonB-linked outer membrane receptor | PG1752 | Transport | 93 kDa | -1.5 | 15 | 10 |
| hypothetical protein | PG0197 | Unknown | 33 kDa | -1.8 | 9 | 5 |
| conserved hypothetical protein; possible TonB-dependent receptor | PG0899 | Transport | 100 kDa | -2 | 8 | 4 |
| outer membrane protein, TonB dependent receptor | PG0637 | Transport | 94 kDa | -2.9 | 52 | 18 |
| phenylalanine/histidine ammonia-lyase | PG0299 | Histidine catabolysis | 55 kDa | -3.3 | 23 | 7 |
| outer membrane protein | PG1901 | Cell envelope | 32 kDa | -3.6 | 29 | 8 |
| beta-galactosidase | PG0598 | Carbohydrate metabolism | 127 kDa | -3.8 | 15 | 4 |
| hypothetical protein | PG1894 | Unknown | 21 kDa | -10.8 | 43 | 4 |
| tonB-linked outer membrane receptor | PG1242 | Transport | 96 kDa | -16 | 80 | 5 |

## Palmitic Acid Assay Shows VimA to Be Involved in the Acylation of Proteins

Several outer membrane proteins are lipid modified in *P. gingivalis* (85). To determine whether the VimA could play a role in lipid modification of proteins *P. gingivalis* was grown in the presence of $^{3}H$ labeled palmitic acid. No change in the acylation profile was observed in the total protein fractions of the wild-type or FLL92 grown to exponential phase (Fig 2.5. B). In the extracellular fraction from FLL92 grown to exponential phase (Fig 2.5. B), a 54 kDa protein was observed in W83 which had 3 times the intensity of that observed in FLL92; while a 27 kDa protein was observed in FLL92 with twice the intensity of W83 (Fig 2.5. C).

TABLE 2.4. Predicted motifs present in missing FLL92 membrane proteins

| | PG0212 (Carboxy-peptidase D) | PG0554 (Hypothetical Protein) | PG0981 (Hypothetical protein) | PG1136 (Branched Chain AA transaminase) | PG1249 (Peptidylarginine deiminase) | PG1605 (Kgp) | PG1722.1 (Hypothetical protein) | PG1768 (RgpA) | PG1857 (Glyceraldehyde 3-phosphate dehydrogenase) |
|---|---|---|---|---|---|---|---|---|---|
| MYR * | 13 | 3 | 11 | 6 | 8 | 49 | 7 | 37 | 7 |
| TYR_PHOS** | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 |
| CK2_PHOS† | 19 | 5 | 14 | 4 | 5 | 29 | 3 | 25 | 8 |
| PKC_PHOS†† | 15 | 5 | 16 | 3 | 6 | 27 | 5 | 20 | 5 |
| CAMP_PHOS‡ | 1 | 0 | 1 | 0 | 1 | 2 | 1 | 2 | 1 |
| ASN_GLYC ‡‡ | 8 | 4 | 0 | 3 | 5 | 15 | 1 | 17 | 3 |
| Amidation § | 1 | 0 | 3 | 0 | 1 | 4 | 0 | 4 | 0 |
| Unique | Zinc_Car¶ | None | None | None | None | DNA-Lig# | None | DNA-Lig | GAPDH## |

(*-N-myristoylation site, **-Tyrosine kinase phosphorylation site, †-Casein kinase II phosphorylation site, ††-Protein kinase C phosphorylation site, ‡-cAMP and cGMP-dependent protein kinase phosphorylation site, ‡‡-N-glycosylation site, §-Amidation site, ¶-Zinc Carboxypeptidase, #-ATP-dependent DNA ligase AMP-binding site. ##-Glyceraldehyde 3-phosphate dehydrogenase active site)

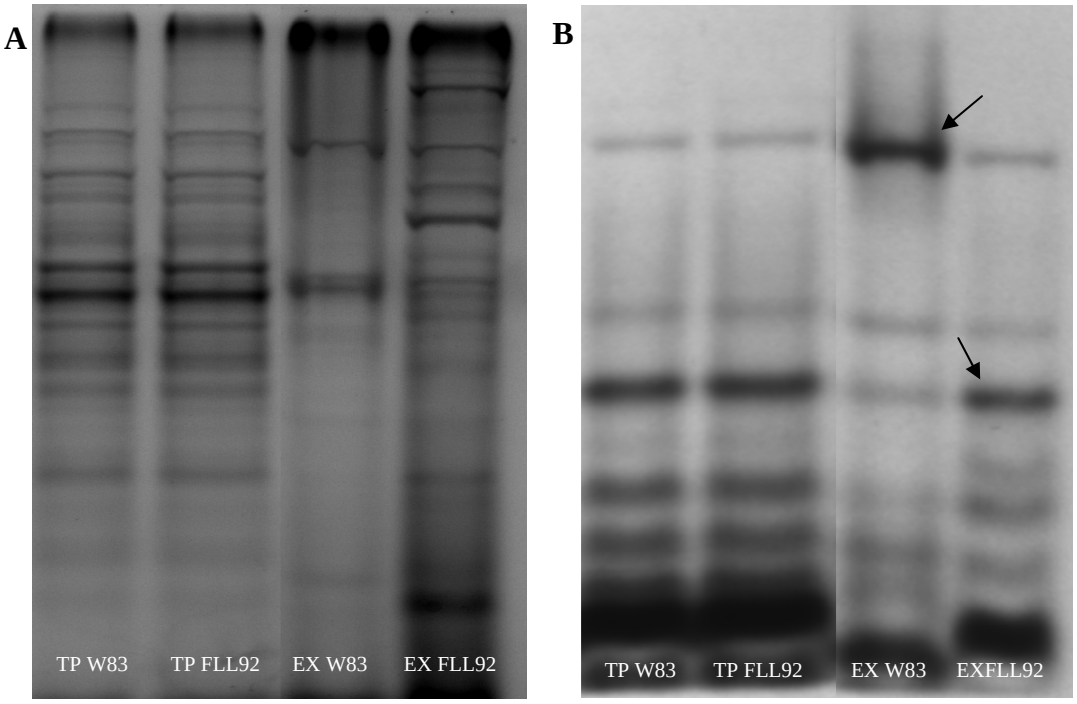


Fig. 2.5. Autoradiography of total protein (TP) and extracellular fractions (EX) of W83 and FLL92 grown overnight in the presence of $^{3}H$ palmitic acid, (A) Brilliant Blue stained SDS Page gel with W83 and FLL92 TP & EX fractions. (B) $^{3}H$ palmitic acid labeled nitrocelluloase membrane with W83 and FLL92 TP & EX fractions.

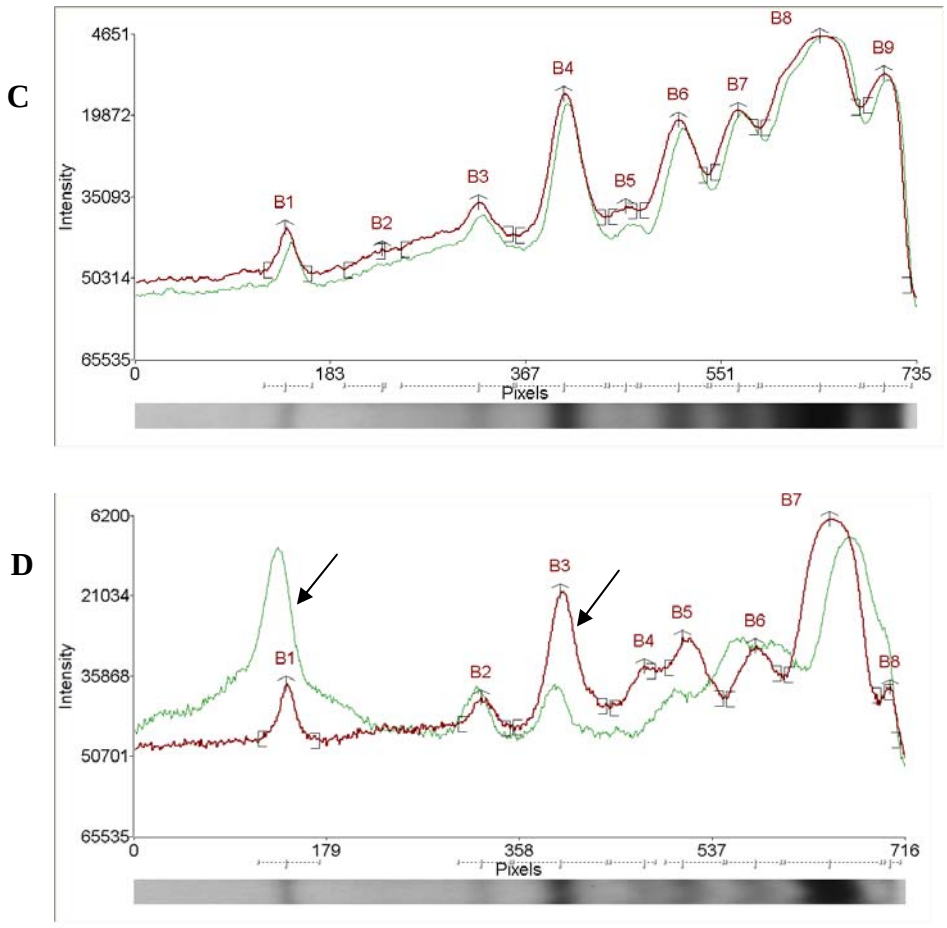


Fig. 2.5. (continued). (C) Densitometric analysis of $^{3}$H lipid labeled proteins of W83 & FLL92 total protein fraction. (D) Densitometric analyis of $^{3}$H lipid labeled proteins of W83 & FLL92 extracellular fraction. A 54 kDa protein (B1) was three times as abundant as that observed in FLL92 and the 27 kDa protein (B3) was twice as abundant in FLL92 compared with W83

## Discussion

Membrane integrity is critical to the survival, adaptability and pathogenicity of bacteria (15,18,86). In this study, we examined the role of VimA on membrane proteins and structures, with a view to further elucidate its role in modulating virulence in *P. gingivalis*. The role of VimA in gingipain maturation, auto-aggregation, hemolysis, hemagglutination, and LPS synthesis has been alluded to in previous reports (77-80); however, its role in membrane biogenesis has not been explored. The unique phenotype of FLL92 led us to hypothesize that VimA plays a significant role in membrane biogenesis of *P. gingivalis* and is integral for anchorage and maturation of membrane proteins.

*In silico* analysis of the VimA protein predicted a structure that showed similarity to the Fem family of proteins. These proteins are found in Gram positive bacteria and are involved in cell envelope biogenesis, particularly in peptidoglycan formation (7,39,72). Interestingly, this protein is not predicted for secretion or cytoplasmic localization, suggesting that it is likely transmembrane or periplasmic. Attempts to localize this protein with antibodies raised against the over-expressed protein in *E. coli* have so far proven unsuccessful. The VimA protein could be classified in the DUF482/CH1444 and/or Acyl CoA N-acyltransferase superfamily. The DUF482/CH1444 is part of the PEP-CTERM system that has sortase-like function and is involved in bacterial exopolysaccharide biosynthesis (23). Proteins exported in this system have conserved C-terminal sorting signals that are recognized by a sortase in Gram positive bacteria (13,14,40,75,76) or an analogous system termed exosortase in Gram negative bacteria (66). The second predicted family (Acyl-CoA N-acyltransferase) is a broad family which includes: N-acetyltransferase, N-myristoyl transferase, autoinducer synthase, FemXAB nonribosomal peptidyltransferases, Ornithine decarboxylase antizyme-like, AstA-like, DUF1122, EF1021-like. The VimA protein is predicted to belong

to the FemXAB nonribosomal peptidyltransferase subfamily. Notably, the VimA is predicted for interaction with the RgpB (score 0.81) in addition to several other proteins. This is consistent with previous reports which demonstrate that the VimA is able to interact with RgpB. Interestingly, several proteins including those coded for upstream and downstream of the *vimA* are predicted for interaction, it is noteworthy that several proteins including PG1605 (Kgp), PG0010 (β-lactamase), PG1101 (Alanyl-tRNA synthase), PG0535 (HtrA), PG1833 (RegT), PG0324 (putative sialidase), and PG1768 (RgpA) that have been previously shown to interact (80) were not predicted for interaction. This data suggests that the VimA is likely a multifunctional protein, possibly forming a complex with those proteins encoded at the recA locus, and may modulate several virulence factors in addition to being involved in the biosynthesis of membrane proteins and structures.

We have demonstrated in previous studies that compared to the wild-type, FLL92 autoaggregates (53). Several reports have suggested that the expression of fimbriae and the absence of a capsule may contribute to auto-aggregation, which in turn increases the organism's ability to form biofilm (11). Visible differences were observed between FLL92 and W83. These differences related to capsular formation and surface morphology. The capsule of FLL92 was irregular and fuzzy in contrast to the solid defined capsule of W83. FLL92 also had a corrugated surface morphology compared to the smooth morphology present in W83. Abundant immunogold particles were observed in the appendage like structures of FLL92; however, few gold particles were incorporated in the few surface related appendages of W83. Interestingly, RT-PCR demonstrated that the *fimA* gene was similarly expressed in both the wild- type and *vimA*-defective isogenic mutant.

The presence of major fimbriae in wild-type W83 was earlier disputed (52), as screening by immunological means such as bacterial agglutination, ouchterlony

immunodiffusion and western blotting were all negative; however, electron microscopy has been used successfully to establish that W83 is poorly fimbriated (24,74). This is in contrast to highly fimbriated strains such as 33277 and 381 which show positive immunological fimbrial results and positive visualization by electron microscopy. In *P. gingivalis*, there are six classifications of the *fimA* gene (I, Ib, II, III, IV and V) (4); with the large majority of patients carrying either type II or IV *fimA*; interestingly, these genotypic differences do no correlate with morphological differences in fimbriae (2). W83 is type IV, whereas 33277 and 381 are type I (52). The relationship between capsular and fimbrial synthesis is at present unknown in *P. gingivalis*, however as has been demonstrated in *Klebsiella pneumoniae* (41, 65), fimbrial function can be impeded by expression of the polysaccharide capsule and can be restored by inhibiting capsular synthesis. Capsular formation may also impede assembly of preformed fimbrial subunits on the bacterial surface (65). This effect in *K. pneumoniae* is due to direct physical interaction as opposed to transcriptional or translational changes; and is in keeping with our own observations which show that the *fimA* transcript is made in the wild-type and the mutant. Therefore, in light of the effect of the VimA mutation on capsular synthesis, fimbrial function and synthesis may no longer be impeded, resulting in the assembly of fimbriae on the surface. The presence of the *fimA* message coupled with undetected FimA via immunoblotting, may also indicate a role for *vimA* in protein synthesis and turnover. Interestingly, these alterations in membrane structure did not affect the organism's ability to form biofilm. Taken together, these findings indicate a critical role for VimA in capsular biogenesis and a likely indirect role in fimbrial synthesis. Further studies are actively being pursued in our laboratory to elucidate the role of the VimA in synthesis and regulation of the capsule and fimbriae.

The corrugated surface morphology of FLL92 coupled with previous reports which demonstrated that though there are more proteins present in the membrane

fraction, other proteins such as RgpA and Kgp are missing (79,80), could suggest that this was likely due to the aberrant anchorage and or expression of proteins on the cell membrane. Our findings revealed twenty proteins that were aberrantly anchored to the cell wall of FLL92, nine proteins that were missing and twenty one proteins which had variable spectral count values. These normalized spectral count values correlate to the relative abundance of these proteins. Of the nine missing proteins, three are involved in proteolysis, these included: Carboxypeptidase D (PG0212), RgpA (1768) and Kgp (1605). Glyceraldehyde 3-phosphate dehydrogenase –GAPDH (PG1857) and peptidyl-arginine deiminase (PG1249) were also missing.

RgpA and Kgp proteinases and adhesins are C terminally processed by Carboxypeptidase D otherwise known as carboxypeptidase CPG70. This protein shares C-terminal sequence similarity to cysteine proteinases indicating a common mechanism for cell surface attachment and secretion (8,81). The absence of this carboxypeptidase on the surface of FLL92 likely explains why RgpA and Kgp are incorrectly modified and inactive, existing in the extracellular milieu, but not anchored to the surface. This is in contrast to the surface bound yet inactive RgpB. GAPDH is a metabolic enzyme of the glycolytic pathway which phosphorylates glyceraldehyde-3-phosphate to generate 1,3 biphosphoglycerate (34). GAPDH has also been shown to exist on the surface of bacterial pathogens; functioning as a novel virulence factor in several bacterial species, by binding host proteins (38,55). In *Trichomonas vaginalis*, this enzyme binds fibronectin, plasminogen and collagen (34) while in enterohemorrhagic *E. coli* and enteropathogenic *E. coli* it binds human plasminogen and fibrinogen (16). In *Candida albicans* this surface associated enzyme binds to laminin and fibronectin (21). Surface bound GADPH of *Streptococcus oralis* has been shown to play a role in *P. gingivalis* colonization. The FimA of *P. gingivalis* is able to bind to GADPH present on the streptococcal surface (46), this interaction is further used to bind to human oral epithelial

cells (31). The function of surface bound GADPH in *P. gingivalis* has not been elucidated however we speculate that it is likely serving to bind host extracellular matrix proteins present within the periodontal pocket for proteolytic degradation by the gingipains. It is also possible that this protein may itself contain a binding receptor for other oral bacteria so as to allow for biofilm formation and colonization of the oral cavity. Peptidyl arginine deiminase (PAD) - catalyzes the deimination of the guanidino group from carboxyl-terminal Arg residues to produce ammonia - is released by *P. gingivalis* with the progression and development of adult-onset periodontitis. PAD provides a protective effect to the bacterium during the acid cleansing cycles of the mouth (68) through the production of ammonia. The activity of PAD distributed among cells, supernatant and vesicles has been shown to be growth dependent. It has also been suggested that the interaction between PAD and Arg-X may arise from Arg-X cleaving anti-adhesive humoral defense peptides containing internal arginyl residues and other arginine carboxyl terminal defense peptides, which are then inactivated by PAD (43,64). It is likely that anchorage of PAD may be dependent on the carboxypeptidase D processing. The reported interaction between between Arg-X and PAD, leads us to hypothesize that the enzymatic capabilities of PAD may be compromised as Arg-X is inactive, thus reducing its efficacy. Further studies are needed to clarify this hypothesis.

When missing proteins were examined for protein motifs using PPsearch - a protein motif identifier software, myristoylation and phosphorylation sites were in great abundance in all of the missing proteins. Of the 20 proteins, 13 were hypothetical proteins, 9 of which had an unknown function. Further, PG0535 (Serine periplasmic protein) which has been shown to interact with VimA is aberrantly expressed on the membrane of the FLL92. This study also confirmed in FLL92 the missing membrane-associated RgpA and Kgp as previously reported (79,80). These findings likely explain the auto-aggregation that is observed in FLL92 and support a role for VimA in anchorage

and maturation of membrane proteins, possibly through myristoylation or phosphorylation. The specific interaction between the VimA and missing or aberrantly expressed proteins is being investigated.

In the vimA defective mutant there is aberrant protein glycosylation (79). It is not known however whether this aberrant glycosylation is specific to certain carbohydrate moieties. To clarify the effect of the *vimA* mutation on glycosylation of outer membrane proteins, lectins were used. Outer membrane proteins glycosylated with Galactose (β 1,3) N-Acetylgalactosamine, N-acetyl-α-D-galactosamine, Galactose (β 1,4) N-Acetylglucosamine, N-acetyl-D-galactosamine and Sialic Acid (N-Acetyl neuramic acid) were affected by the *vimA* mutation. Whether this due to the glycosylation of aberrantly expressed proteins (i.e. proteins that are not present on the wild-type membrane but present on the membrane of FLL92) or proteins that are incorrectly processed as is the case with RgpB is still an active area of exploration. These findings suggest that the VimA plays a role in the correct glycosylation of several membrane proteins.

Since several outer membrane proteins are lipid modified in *P. gingivalis* (71), and in keeping with the likely role of the VimA protein as a possible acyl transferase, we incubated W83 and FLL92 with $^{3}H$ labeled palmitic acid (lipid donor). The expression profile of lipid modified proteins in the wild-type and mutant was unchanged. This suggests that the VimA is either not involved in the acylation of outer membrane proteins or that there is a redundant mechanism of protein acylation. To this end, PG1355 (putative acyltransferase) was upregulated (3.5 fold change) in FLL92 (microarray data – unpublished) compared to the mutant. Interestingly, densitometric analysis of the extracellular fractions of W83 and FLL92 for lipid modified proteins revealed a 54 kDa protein that was 3 times more abundant in W83 than was observed in FLL92. We also observed a 27 kDa protein that was twice as abundant in FLL92 as present in W83. The above findings may indicate a likely role for VimA in the proper acylation of a 54 kDa

extracellular protein; however, we have not ruled out the possibility that this protein is a dimer of the 27 kDa protein observed in the extracellular fraction of FLL92. Several reports have demonstrated that acylation is important for protein folding, protein-protein interaction as well as ligand induced conformational changes (5,26,54). It is possible that correct acylation of the 27kDa extracellular protein leads to dimerization. In the *vimA* mutant, aberrant acylation of the 27 kDa extracellular protein may occur, resulting in higher levels of monomeric protein form. Further studies in our laboratory are seeking to clarify this position.

The expression of additional proteins attributed to membrane biosynthesis and transport in the VimA mutant suggest that several post translational modification pathways may be affected by this protein. These modifications could include myristoylation and glycosylation. Co-translational modifications of proteins could be attributed to their involvement in signal transduction pathways as molecular switches associated with glycine rich protein motifs. Myristoylation of proteins are reported to be involved in Type III secretory system in prokaryotes (51)

The missing and aberrantly expressed proteins in FLL92 suggest that VimA could have sorting/transport functions. VimA could also function as an acyl CoA N-acyltransferase enzyme involved with protein modification/anchor age of membrane proteins. In conclusion, our results highlight a putative central role for the VimA protein in protein modification and transport, possibly triggering cascade reactions that may modulate the virulence potential of *P. gingivalis.* The putative multiple functions of the VimA protein needs further clarification.

**Acknowledgements**

This work was supported by Loma Linda University and Public Health Grant DE13664 and DE019730 from NIDCR (to H.M.F). We thank Prof. Masatoshi Inukai for the

Globomycin antibiotic, Dr. Hua Xie for the FimA antibody, and Dr. Danielo Boskovic for helpful comments concerning the acylation studies.

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CHAPTER THREE

# VimA IS INVOLVED IN PEPTIDOGLYCAN BIOGENESIS AND PROTEIN SECRETION

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Running title: VimA is a putative acyl transferase involved in peptidoglycan synthesis

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**Summary**

Several outer membrane structures and secreted proteins contribute to the invasive potential and virulence of *P. gingivalis*. We have previously demonstrated that the VimA protein of *Porphyromonas gingivalis* is a multifunctional protein involved in several cellular processes, including gingipain maturation, fimbrial synthesis, lipid polysaccharide polymerization and anchorage of outer-membrane proteins. *In silico* analysis predicts the VimA protein is part of the FemXAB non-ribosomal peptidyltransferase family which is involved in peptidoglycan synthesis. To further characterize the VimA, we evaluated its role in peptidoglycan biogenesis and protein sorting. The peptidoglycan of the *vimA* mutant and wild-type W83 were isolated and purified from cells grown to log and stationary growth phase and examined using Atomic force microscopy and transmission electron microscopy. Hydrolytic enzymes assays were used to determine differences in the rate of hydrolysis between strains. Mass Spectrometry was used to identify aberrantly expressed and missing extra-cellular proteins. Our results indicated structural and compositional differences in the peptidoglycan of W83 when compared to FLL92. The rate of hydrolysis with lysostaphin was higher in W83 than in FLL92. Notably, sixty-eight proteins were present only in the extracellular fraction of FLL92. In the same fraction, there were 16 proteins present both in the wild-type and mutant; however, they varied in abundance. These observations suggest that the VimA protein is likely involved in peptidoglycan synthesis, and corroborates our previous report, which suggests a role for VimA in protein sorting.

## Introduction

*Porphyromonas gingivalis* is a black pigmented, Gram negative, asaccharolytic, anaerobic, coccobacilli, which is an important etiologic agent of periodontal disease (6,8,11,31). Strains from *P. gingivalis* are classified as either invasive or non-invasive. Invasive strains have been shown to possess more pathogenic activity *in vivo* and *in vitro* than the non-invasive strains (9,12,20,22). Furthermore, a variety of bacterial cell surface components including: capsule, polysaccharides, proteases (gingipains), hemagglutinin, lipopolysacharides, major outer membrane proteins and fimbriae contribute to cell adherence and virulence of *P. gingivalis* (26,41,43). These bacterial surface components may also play a role in resisting phagocytosis by leukocytes (29).

Peptidoglycan, a constituent of the bacterial cell membrane, has been demonstrated to induce several host inflammatory responses. This heteropolymer is comprised of glycan strands cross-linked through peptide chains. These chains are of two types – stem peptides that are linked to the glycan chain, and a cross-bridge that links stem peptides. There is significant diversity in the composition and sequence of the cross-bridges and stem peptides across bacterial species (5)

*P. gingivalis* contains a 51kDa *murC* gene, which is a homologue of the *E. coli* peptidoglycan synthesis gene, and catalyses the first step in the biosynthesis of the cell wall peptidoglycan (2). An examination of eighteen vesicle preparations from *P. gingivalis* identified muramic acid as present in each preparation. These muramic acid moieties were regarded as low molecular mass compounds, as they were soluble in TCA [cross-linked peptidoglycans and muramic acid is reported to be insoluble in TCA]. This led the authors to suggest that the products of cell wall turnover – removal of peptidoglycan fragments from pre-existing cell wall (10), exert turgor on the outer membrane, which results in the herniation and blebbing of the outer membrane (44) – formation of vesicles. Several alternate peptidoglycan derived models of vesicle

formation have also been proposed (7,24). The inflammatory reaction produced in the host to vesicles may result in part from the presence of muramyl peptides and lipopolysacharides (44).

The human proteins (Nucleotide-binding Oligomerization Domain) Nod1 and Nod2 – involved in the intracellular recognition of bacterial products - though initially thought to recognize bacterial lipopolysacharides, is now believed to specifically recognize peptidoglycans. Whereas Nod1 recognizes gamma-D-glutamyl-meso-DAP (found primarily in gram negatives), Nod2 recognizes muramyl dipeptides (MDP) (14).Two common gene polymorphisms of CARD15 - encodes the Nod2 protein – in periodontitis patients did not reveal statistical differences between the control group and the periodontitis group, suggesting no role for these mutations in adult periodontitis (25). Furthermore, in human embryonic kidney cells stimulated with heat killed *P. gingivalis*, weak NOD1 and NOD2 stimulatory activity was observed when compared with other oral bacteria, suggesting that weak NOD stimulation might be important for the survival of *P. gingivalis* in the periodontal pocket (32). Peptidoglycan from *P. gingivalis* has however been demonstrated to induce upregulation of CD14 and CD16 in dendritic cells (DCs) via TLR2 signaling (21). These DCs release higher levels of IL-8 and RANTES at lower doses than DCs stimulated with fimbriae. Peptidoglycan from *P. gingivalis* has also been shown to elicit the production of IL-6 from mouse peritoneal macrophages, and induces cell death in silkworm tissues via reactive oxygen species (ROS) production (19).

In our previous studies using *In silico* analysis, the predicted structure of VimA was similar to the Fem family of proteins (33). These proteins are found in Gram positive bacteria and are involved in cell envelope biogenesis, particularly in peptidoglycan formation (4,27,38). Several superfamily prediction software also classified this protein as belonging to the DUF482/CH1444 (contain determinants for C terminal sorting of proteins) and Acyl-CoA N-acyltransferase superfamily (broad family which includes the

FemXAB nonribosomal peptidyltransferases). VimA also interacts with PG1101 (Alanyl-tRNA synthase) (40), which is likely involved in peptidoglycan synthesis. These studies led us to speculate that VimA is a possible acyl transferase involved directly in peptidoglycan biogenesis. To confirm this hypothesis, we examined the morphology of the peptidoglycan from W83 and FLL92, and utilized several enzymatic assays to elucidate the possible changes in the peptidoglycan. Mass spectrometry was used to determine whether the abnormal secretion protein profile observed in FLL92 was limited to the outer membrane proteins.

## Materials and Methods

### Bacterial Strains and Growth Conditions

*P. gingivalis* strains (W83, ATCC 33277 and FLL92) and *Enterococcus faecalis* were grown in either Brain Heart Infusion (BHI) broth (Difco Laboratories) supplemented with cysteine (0.1%), vitamin K (0.5 µg/ml) and hemin (5 µg/ml) (BHIKH) or in Trypticase Soy Broth containing menadione and hemin (TSBKH). Solid medium was prepared by supplementation with 1.5% agar and 5% defibrinated sheep blood (Hemostat laboratories). All cultures, unless otherwise stated, were incubated at 37˚C in an anaerobic chamber (Coy Manufacturing) in 80%$N_2$, 10% $H_2$ and 10% $CO_2$. Growth rates were determined spectrophotometrically at 600nm (optical density).

### Complementation of *vimA*

PCR mediated gene replacement was used to complement the *vimA* defect. In brief, the ORF of vimA was amplified using primers specific for the *vimA.* The amplified fragment was purified by agarose gel electrophoresis, then electroporated into electro competent FLL92 cells grown to log phase ($OD_{600}$ of 0.6). Electroporated cells will be incubated for 12 hours in 1ml of broth then plated on BHIHK-blood plates. Plates were

then screened after 8 days for black-pigmented colonies. These colonies were subsequently evaluated for the presence of the uninterrupted *vimA* gene using PCR.

## Peptidoglycan Isolation from W83 and FLL92

*P. gingivalis* peptidoglycan was prepared using the modified method previously reported by Isahii (19). In brief, 200ml of *P. gingivalis* W83 and FLL92 cultures were centrifuged (9000xg, 10mins at 10C), and the precipitates resuspended in water. Trichloroacetic acid (10%) was added to the sample and the mixture was incubated at 4º for 1 hour. The sample was centrifuged (9000xg, 5 min, 10ºC) then washed three times in water. The precipitate was incubated at 100ºC for 1 hour after being suspended in 50mM sodium acetate buffer (pH 5.3) containing 8% SDS. This was followed by an overnight incubation at room temperature. After centrifugation (43,000xg, 1 hour, 12ºC), the sample was washed three times in water and resuspended in Tris HCL containing 2% SDS and proteinase K (50µg/ml) at 37°C for 12 hours.

## Preparation of Murein Sacculi

Murein Sacculi was prepared from FLL92 and W83 using the method of Yao (42). Briefly, cells were grown to $OD_{600}$ of 0.7 representing log phase and 1.5-1.6 representing stationary phase in 2000mls of BHIKH media. Cultures were then centrifuged at 6000xg for 30 minutes, washed twice then resuspended in phosphate buffered saline (pH 7.0) containing 1mM magnesium chloride, then washed twice. The suspension was then boiled in 2% wt/vol SDS for 3 hours and left overnight at room temperature for 48 hours, after which it was centrifuged for 1 hour at 150,000xg (25ºC) to pellet the Sacculi. Sacculi were washed three times in deionized water at room temperature, then dialyzed overnight in one liter of deionized water.

## Lytic Enzyme Assays

Several peptidoglycan hydrolytic enzymes, including muramidase (Sigma), and lysostaphin (Sigma) were used to determine the susceptibility of peptidoglycan and the murein sacculi to hydrolysis. In brief, peptidoglycan was suspended in 0.1 M potassium hydrogen phosphate, pH 7.3 to an approximate absorbance of 0.7 at 580 nm, in a 1cm light path. One hundred µg/ml was added to the peptidoglycan suspension at zero time and incubated at 37ºC. The absorbance at 580nm was followed for 24 hour and the percent hydrolysis was calculated employing the absorbance of an untreated, peptidoglycan control (3).

## Microscopic Examination

Atomic Force Microscopy (AFM) and transmission Electron microscopy (TEM) were used to provide detailed visuals of the morphology of the murein sacculi. AFM was done using treated and untreated murein sacculi, isolated from W83 and FLL92 stains grown to stationary (OD 1.5-1.8) and exponential (OD 0.6 – 0.7) growth phase. Samples were washed twice in 0.1M PBS buffer pH 7.4 and mounted on a cover slide, then allowed to air dry. AFM was performed using the Innova Scanning Probe Microscope (Veeco Instruments Inc) in air using the tapping mode. Images were captured in the height mode or peak force error (35). Transmission Electron Microscopy was performed using the Philips Tecnai 12 TEM as per the method of Hyatt (18). Briefly, sacculi were absorbed to 400- mesh carbon and Formvar coated EM grids. A drop of 2% (wt/vol) uranyl acetate was used to float the grid for 15s to negatively stain the sample, this was followed by microscopy using a Philips Tecnai 12 operated under standard conditions with the cold trap in place (42).

## Bioinformatics Analysis of VimA

ClustalW (ebi) was used to compare the VimA protein and gene sequences to the FemA, FemB and FemX, while CLC Main Workbench 5 was used to align the sequence of the missing extracellular proteins derived from Mass spectrometry.

## Cell Fractionation

Extracellular fractions were prepared from cell-free culture fluid precipitated with 60% acetone (-20ºC) (40) from W83 and FLL92. The protein pellet was resuspended in 7 ml 100 mM Tris-HCL buffer (pH 7.4) in the presence of 1 mM *N-p*-tosyk-L-lysine chloromethyl ketone (TLCK), dialyzed for 48 hours against the same buffer then stored at -20ºC until used.

## Digestion of *P. gingivalis* Wild-type and FLL92 Proteins

Extracellular proteins were run on a 10% bis-Tris gel (Invitrogen, Carlsbad, CA) in 1X MOPS running buffer for 1.5 cm, then visualized by staining with SimplyBlue safe stain (Invitrogen) (16). After destaining in water, the gel was cut into 1-2 mm slices. Gel slices were subsequently dehydrated in acetonitrile and dried in a vacuum centrifuge for 30 minutes. The gel slices were incubated for 1 hour at 60ºC in a solution containing 20 µl of 20mM Dithiothreitol in 100mM $NH_4HCO_3$ (enough to cover the gel pieces). The Dithiothreitol solution was replaced with an alkylating solution (20 µl of 200 mM iodoacetamide in 100 mM $NH_4HCO_3$) after cooling the proteins to room temperature. Gel slices were further incubated at ambient temperature for 30 minutes in the dark, followed by two washes with 150 µl of 100 mM $NH_4HCO_3$, then finely minced with a flame sealed polypropylene pipette tip, dehydrated by the addition of acetonitrile, and vacuum dried. Following an overnight incubation of the gel pieces with 20 µl digestion buffer [1 µl of

mass spectrometry (MS)-grade trypsin (www.promega.com) in 50 mM acetic acid with 1 µl of 100 mM $NH_4HCO_3$], the digestion reaction was stopped with 10 µl of 5% formic acid. After transferring the digest solution (aqueous extraction) to a 0.65 ml siliconized tube, 30 µl of 50% acetonitrile with 0.1% formic acid was added, the mixture was vortexed for 3 minutes, centrifuged and then sonicated for 5 minutes. The process was repeated and both extractions pooled and concentrated to 10 µl in a vacuum centrifuge. Peptide extraction was accomplished using standard $C_{18}$ ZipTip technology following the manufacturer's directions (Millipore, Bedford, MA).

## MS and Data Analysis

An LCQ Deca XP Plus system (www.thermo.com) with nano-electrospray technology (www.newobjective.com) consisting of a reverse phase $C_{18}$ separation of peptides on a 10 cm by 75µm capillary column using Microm Magic RP-18AQ resin (www.michrom.com) with direct electrospray injection to analyze the extracted peptides from each gel piece (16). A four part protocol was used for the MS and MS/MS analyses, this included one full MS analysis (from 450 to 1750 m/z) followed by three MS/MS events using data-dependent acquisition, where the most intense ion from a given full MS scan was subjected to collision-induced dissociation, followed by the second and third most intense ions. The nanoflow buffer gradient was extended over 45 minutes in conjunction with the cycle repeating itself every 2 seconds, using a 0-60% acetonitrile gradient from buffer B (95% acetonitrile with 0.1% formic acid) developed against buffer A (2% acetonitrile with 0.1% formic acid) at a flow rate of 250 to 300 nl/min, with a final 5 minute 80% bump of buffer B before re-equilibration. In order to move the 20 µl sample from the autosampler to the nanospray unit, flow stream splitting (1:1000) and a Scivex 10 port automated valve (Upchurch Scientific, Oak Harbor, WA) together with a Michrom nanotrap column was used. The spray voltage and current were set at 2.2kV and 5.0µA,

with a capillary voltage of 25V in positive ion mode. 160°C was used as the spray temperature for peptides. Data collection was achieved using the Xcalibur software (Thermo Electron), then screened with Bioworks 3.1. MASCOT software (www.matrixscience.com) was used for each analysis to produce unfiltered data and out files. Statistical validation of peptide and protein findings was achieved using X! TANDEM (www.thegmp.org) and SCAFFOLD 2 meta analysis software (www.proteomesoftware.com). The presence of two different peptides at a probability of at least 95% was required for consideration as being positively identified. Confirmation of individual peptide matches was achieved using the BLAST database (www.oralgen.lanl.gov)

## Results

### VimA is Closely Related to the FemX of *Weisella viridescens*

The phylogeny suggests that VimA (0.52) is closer in relation to FemX (0.45) than FemB (0.32) and FemA (0.29) (FemA). Additionally, several consensus sequences were identified in both VimA and FemX, these included1: RY*E**RO, KL****RDG****S, and EG*LL (Fig. 3.2.).

### VimA Affects the Secretion of Several Extracellular Proteins

Mass spectrometric analysis was done to determine whether the differential protein profile observed previously in FLL92 (33), was unique to the membrane proteins or whether this mutation also affected the extracellular protein fraction. Mass Spectrometry of membrane fractions of W83 and FLL92 identified 15 proteins that were present in the extracellular fraction but absent in W83 (Table 3.1.) and 68 proteins that were present in the extracellular fraction of FLL92 (Table 3.2.) but absent in W83. The majority of aberrantly expressed proteins were predicted for involvement in energy

metabolism, while the remainder was predicted to be involved in proteolysis, protein binding and transport, DNA metabolism or were hypothetical. Of the fifteen proteins identified as missing, six were predicted to play a role in cell envelope biogenesis; while two - PG0468 and PG1357 were predicted to be involvement in protein targeting and transport. Six of these proteins were hypothetical. Seven extracellular proteins were identified in FLL92 as having spectral count values with negative fold changes (fc) of 1.2 or greater (Fig 3.3.) - RgpA (PG1768, fc 1.2), Kgp (PG1605, fc 6.6), Carboxypeptidase D (PG0212, fc 28), Peptidylarginine deiminase (PG1249, fc 12.1), Hemmaglutinin (PG1602, fc 22.3), TonB-dependent OM receptor (PG0170, fc 285), and a hypothetical protein (PG0554, fc 50.7).

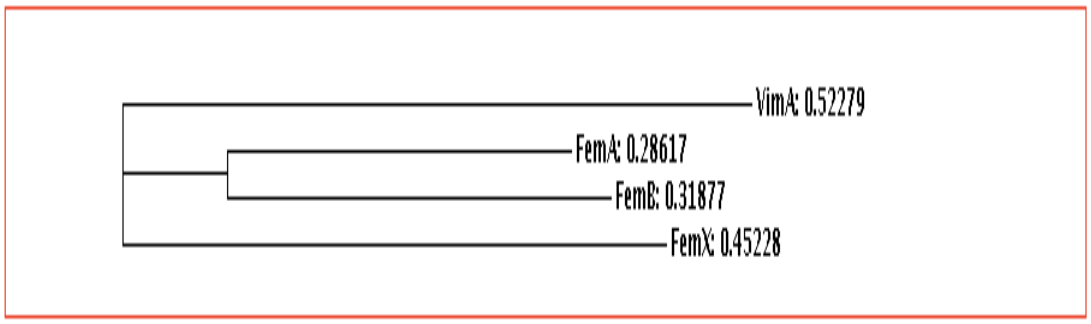


Fig 3.1. Phylogram of VimA, FemA, FemB & FemX shows a close relationship of VimA to FemX

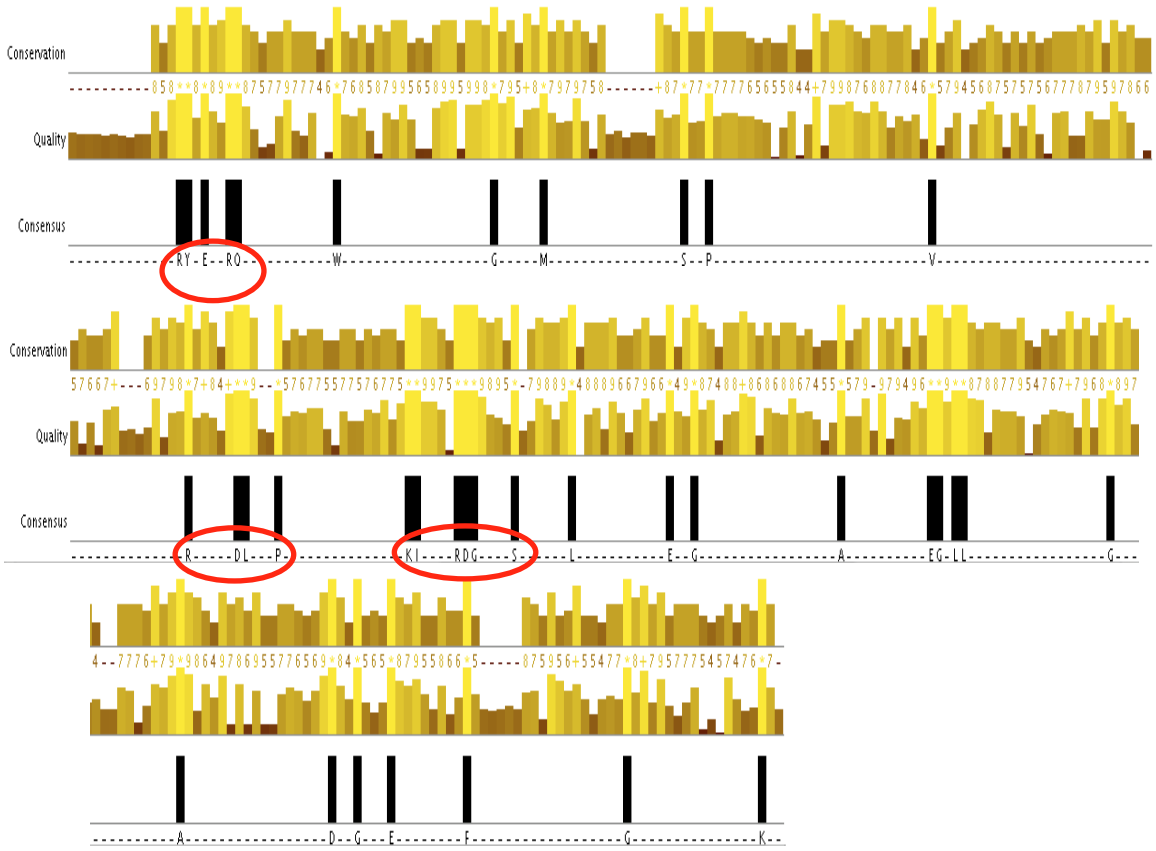


Fig 3.2. Several consensus sequences are present in both VimA and FemX

The terminal residues from the 15 missing extracellular proteins were examined to determine whether a common sequence was present in each. No common domain was present in all these proteins (Fig 3.4)**.** Using PPSearch (http://www.ebi.ac.uk/Tools/ppsearch/), several common domains were identified in the fifteen extracellular proteins identified by MS analysis (Table 3.4.). Casein Kinase II phosphorylation motifs, Protein Kinase C motifs and myristolation motifs were most abundant.

## Complementation of the VimA Defect

The VimA defect was complemented using a PCR mediated replacement strategy. Several black pigmented colonies were identified on blood agar plates after electroporation and incubation with the ORF of VimA, these were designated FLL92C, FLL92B and FLL92D. FLL92B and FLL92D was further characterized and shown to have the characteristic phenotype of W83. FLL92C became hemolytic and black pigmented much later than wild-type W83 (+5 days) and autoaggregated when incubated in BHI broth. Notable, its gingipain activity was marginally higher than FLL92 in log phase. The VimA gene from this strain was sequenced in order to determine whether a mutation in the gene accounted for this phenotype. Two mutations were observed in FLL92C at positions 5 and 6, where CC was replaced by AG. The translated product of this gene contained valine rather than isoleucine at the third amino acid position.

Table 3.1. MS analysis of aberrantly expressed proteins in FLL92 extracellular fraction

| Identified Proteins (68) | Accession # | Putative Role/Function | M.W. | W83 S.C.V | FLL92 S.C.V |
|---|---|---|---|---|---|
| Hypothetical protein | PG0027 | Unknown | 25 kDa | 0 | 2 |
| UDP-3-O-acyl-GlcNAc deacetylase/(3R)-hydroxymyristoyl-acp-dehydratase | PG0060 | Fatty acid biosynthesis | 52 kDa | 0 | 3 |
| Nitrogen assimilation regulatory protein (immunoreactive 47 kD antigen PG120) | PG0136 | Transcription | 47 kDa | 0 | 12 |
| Endothelin converting enzyme/neprilysin (PepO) | PG0146 | Proteolysis | 79 kDa | 0 | 2 |
| NADH oxidase/peroxidase | PG0160 | Energy metabolism | 104 kDa | 0 | 7 |
| Probable outer membrane protein (Omp85 analog) | PG0175 | Cell envelope | 102 kDa | 0 | 5 |
| Uroporphyrinogen-III synthase | PG0185 | Biosynthesis of cofactors | 28 kDa | 0 | 7 |
| Hypothetical protein | PG0227 | Unknown | 11 kDa | 0 | 4 |
| Translation initiation factor IF-2 | PG0230 | Translation | 108 kDa | 0 | 2 |
| Conserved hypothetical protein | PG0264 | Unknown | 39 kDa | 0 | 4 |
| Polyferredoxin | PG0276 | Energy metabolism | 30 kDa | 0 | 2 |
| Probable dipeptidyl peptidase | PG0291 | Proteolysis | 101 kDa | 0 | 2 |
| Conserved hypothetical protein | PG0293 | Unknown | 15 kDa | 0 | 3 |
| DNA-mismatch repair protein | PG0350 | DNA metabolism | 95 kDa | 0 | 9 |
| DNA-directed RNA polymerase subunit beta | PG0360 | Transcription | 142 kDa | 0 | 2 |
| ATP-dependent DNA helicase | PG0381 | DNA metabolism | 82 kDa | 0 | 4 |
| ATP-dependent ClpX-related protease | PG0382 | Proteolysis | 46 kDa | 0 | 16 |
| Ferredoxin oxidoreductase beta subunit | PG0394 | Energy metabolism | 37 kDa | 0 | 2 |
| Conserved hypothetical protein | PG0411 | Unknown | 52 kDa | 0 | 2 |
| Conserved hypothetical protein | PG0452 | Unknown | 54 kDa | 0 | 49 |
| Pyruvate ferredoxin/flavodoxin oxidoreductase | PG0498 | Energy metabolism | 132 kDa | 0 | 2 |
| Hypothetical protein | PG0510 | Hypothetical | 34 kDa | 0 | 5 |
| Beta-galactosidase | PG0598 | Energy metabolism | 127 kDa | 0 | 11 |
| DNA topoisomerase I | PG0680 | DNA metabolism | 90 kDa | 0 | 17 |
| Acyl-CoA dehydrogenase (coenzyme A dehydrogenase) | PG0696 | Metabolic process | 65 kDa | 0 | 2 |
| Probable Xaa-Pro dipeptidase | PG0795 | Proteolysis | 44 kDa | 0 | 4 |
| Beta-galactosidase | PG0799 | Energy metabolism | 116 kDa | 0 | 2 |
| Translation elongation factor G protein | PG0832 | Translation | 80 kDa | 0 | 2 |
| Calcium ion-transporting ATPase | PG0838 | Transport & binding | 118 kDa | 0 | 2 |
| Threonyl-tRNA synthetase | PG0888 | Translation | 75 kDa | 0 | 7 |
| D-lysine 5,6-aminomutase alpha subunit | PG0955 | Energy metabolism | 57 kDa | 0 | 6 |
| Butyryl-CoA dehydrogenase | PG0958 | Fatty acid metabolism | 42 kDa | 0 | 7 |
| Alanine racemase; N-acetylymuramoylalanyl-D-glutamate-2,6,-diaminopimelate-D-alanine-D-alanine ligase | PG0976 | Cell envelope | 92 kDa | 0 | 2 |
| Conserved hypothetical protein | PG0981 | Unknown | 107 kDa | 0 | 9 |

| | | | | | |
|---|---|---|---|---|---|
| Ribonucleotide reductase alpha subunit | PG1010 | DNA replication | 96 kDa | 0 | 6 |
| Probable long-chain fatty-acid-Coenzyme A ligase (long-chain acyl-CoA synthetase) | PG1028 | Metabolic processing | 69 kDa | 0 | 3 |
| Transferase protein | PG1029 | Transposon functions | 43 kDa | 0 | 7 |
| Xaa-Pro aminopeptidase | PG1071 | Proteolysis | 67 kDa | 0 | 14 |
| Hypothetical protein | PG1089 | Unknown | 80 kDa | 0 | 17 |
| GTP-binding protein (possible membrane protein) | PG1097 | Signal transduction | 66 kDa | 0 | 2 |
| Conserved hypothetical protein | PG1128 | Unknown | 47 kDa | 0 | 5 |
| Thiol protease (PrtT related) | PG1251 | Proteolysis | 93 kDa | 0 | 2 |
| O-succinylbenzoate--CoA ligase | PG1330 | Biosynthesis of cofactors | 40 kDa | 0 | 9 |
| Magnesium-protoporphyrin O-methyltransferase; cobalamin biosynthesis protein N | PG1359 | Biosynthesis of cofactors | 163 kDa | 0 | 2 |
| Aminomethyltransferase (glycine cleavage system T protein) | PG1364 | Energy metabolism | 40 kDa | 0 | 16 |
| Nicotinate-nucleotide pyrophosphorylase (quinolinate phosphoribosyltransferase) | PG1377 | Biosynthesis of cofactors | 30 kDa | 0 | 4 |
| Bacteroides aerotolerance operon protein, batD | PG1385 | Adaptations to atypical conditions | 67 kDa | 0 | 2 |
| Fumarate reductase/succinate dehydrogenase flavoprotein subunit | PG1413 | Energy metabolism | 72 kDa | 0 | 7 |
| Cell division protein (ATPase) | PG1430 | Cell division | 96 kDa | 0 | 2 |
| Hypothetical protein | PG1448 | Unknown | 15 kDa | 0 | 21 |
| Conserved hypothetical protein | PG1496 | Transport | 99 kDa | 0 | 2 |
| ABC transporter, ATP-binding protein, MsbA family; MSD-NBD fusion protein | PG1497 | Protein transport & binding | 70 kDa | 0 | 4 |
| Hypothetical protein | PG1504 | Protein binding | 53 kDa | 0 | 2 |
| Immunoreactive 46 kDa antigen PG99 | PG1572 | Unknown | 46 kDa | 0 | 6 |
| Enolase (phosphopyruvate hydratase)(2-phosphoglycerate dehydratase) (laminin binding protein) | PG1593 | Energy metabolism | 46 kDa | 0 | 22 |
| Urocanate hydratase | PG1630 | Energy metabolism | 74 kDa | 0 | 2 |
| Na+/H+-exchanging protein (Na+/H+ antiporter) | PG1634 | Transport & binding | 49 kDa | 0 | 4 |
| Polyphosphate kinase | PG1640 | Polyphosphate biosynthesis | 81 kDa | 0 | 3 |
| 30S ribosomal protein S8 | PG1677 | Translation | 15 kDa | 0 | 4 |
| 30S ribosomal protein S3 | PG1684 | Translation | 28 kDa | 0 | 2 |
| 50S ribosomal protein L2 | PG1687 | Translation | 30 kDa | 0 | 3 |
| 30S ribosomal protein S7 | PG1693 | Translation | 18 kDa | 0 | 2 |
| Hypothetical protein | PG1783 | Cell redox, homeostasis | 39 kDa | 0 | 2 |
| LPS-modified surface protein P59 | PG1838 | Cell envelope | 61 kDa | 0 | 38 |
| Hypothetical protein | PG1867 | Unknown | 51 kDa | 0 | 19 |
| Hypothetical protein | PG1899 | Hypothetical | 27 kDa | 0 | 6 |
| Conserved hypothetical protein | PG1927 | Unknown | 214 kDa | 0 | 3 |
| Excinuclease ABC subunit A | PG1934 | DNA metabolism | 106 kDa | 0 | 6 |

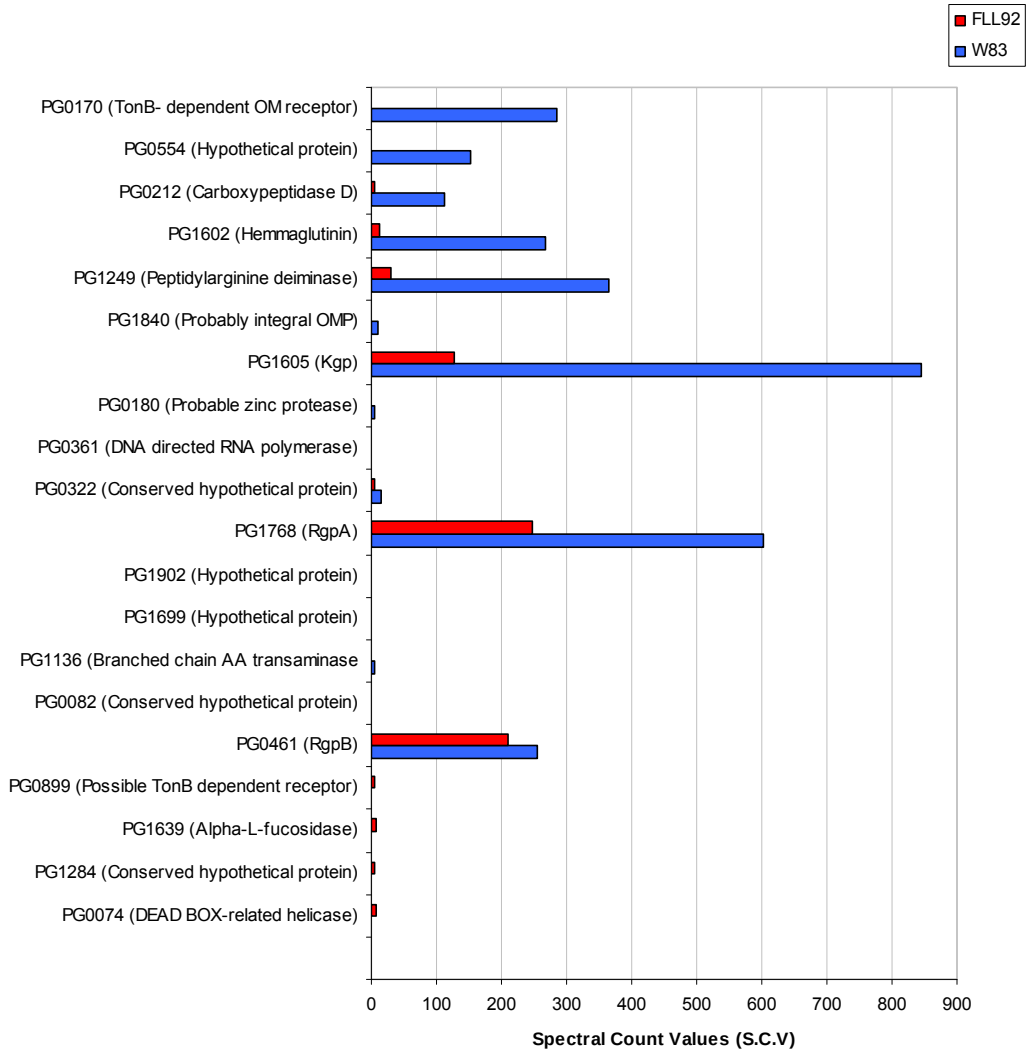


Fig. 3.3. Extracellular proteins with different S.C.V

Table 3.2. MS analysis of missing proteins in FLL92 extracellular fraction

| Identified Proteins (15) | Accession # | Putative Role/Function | M.W. | W83 S.C.V | FLL92 S.C.V |
|---|---|---|---|---|---|
| Receptor antigen B | PG0171 | Cell envelope | 56 kDa | 231 | 0 |
| Conserved hypothetical protein | PG0375 | Translation | 144 kDa | 5 | 0 |
| Preprotein translocase subunit A protein | PG0468 | Protein targeting | 126 kDa | 5 | 0 |
| Glutamine-hydrolyzing carbamoyl-phosphate synthase large subunit | PG0484 | Arginine biosynthesis | 120 kDa | 3 | 0 |
| Hypothetical protein | PG0552 | Unknown | 37 kDa | 10 | 0 |
| Hypothetical protein | PG0592 | Unknown | 45 kDa | 29 | 0 |
| Heme-binding protein/peripheral outer membrane chelatase | PG0602 | Cell envelope | 33 kDa | 10 | 0 |
| Outer membrane protein | PG0626 | Cell envelope | 42 kDa | 10 | 0 |
| TonB-dependent receptor HmuY | PG1357 | Transport & binding | 16 kDa | 9 | 0 |
| Probable integral outer membrane protein P20 | PG1592 | Cell envelope | 24 kDa | 2 | 0 |
| Glyceraldehyde 3-phosphate dehydrogenase | PG1857 | Energy Metabolism | 36 kDa | 2 | 0 |
| Conserved hypothetical protein | PG1875 | Unknown | 123 kDa | 3 | 0 |
| Hypothetical protein | PG1894 | Unknown | 21 kDa | 2 | 0 |
| Outer membrane protein | PG1901 | Cell envelope | 32 kDa | 7 | 0 |
| Conserved hypothetical protein | PG1938 | Unknown | 61 kDa | 7 | 0 |

Table 3.3. Predicted post translational motifs in missing FLL92 extracellular proteins

| Proteins | MYR * | TYR_ PHO | CK2_ PHO | PKC_ PHO | CAMP_ PHOS | ASN_ GLYC | Amid ation | Unique |
|---|---|---|---|---|---|---|---|---|
| PG0171 | 4 | 0 | 6 | 0 | 0 | 2 | 0 | 0 |
| PG0375 | 18 | 0 | 18 | 15 | 0 | 22 | 0 | PTS-HPR Rib S2 |
| PG0468 | 7 | 0 | 20 | 23 | 3 | 4 | 4 | ATP_GTP_A SECA |
| PG0484 | 15 | | 17 | 7 | 1 | 3 | 0 | CPSASE 1 CPSASE 2 |
| PG0552 | 6 | 1 | 3 | 1 | 0 | 1 | 0 | 0 |
| PG0592 | 5 | 0 | 0 | 5 | 1 | 3 | 0 | 0 |
| PG0602 | 3 | 0 | 2 | 4 | 0 | 2 | 0 | 0 |
| PG0626 | 8 | 1 | 5 | 5 | 0 | 0 | 0 | OMPA_1 |
| PG1357 | 2 | 0 | 1 | 6 | 0 | 0 | 1 | ATP_GTP_A |
| PG1592 | 8 | 0 | 2 | 4 | 0 | 1 | 0 | 0 |
| PG1615 | 4 | 0 | 5 | 3 | 2 | 2 | 0 | 0 |
| PG1857 | 7 | 0 | 8 | 5 | 1 | 3 | 0 | GAPDH |
| PG1875 | 16 | 0 | 17 | 19 | 2 | 7 | 0 | RGD |
| PG1894 | 5 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| PG1901 | 1 | 0 | 3 | 5 | 1 | 2 | 1 | 0 |
| PG1938 | 23 | 0 | 5 | 8 | 0 | 4 | 0 | 0 |

(MYR -N-myristoylation site, TYR_PHOS -Tyrosine kinase phosphorylation site, CK2_PHOS -Casein kinase II phosphorylation site, PKC_PHOS - Protein kinase C phosphorylation site, CAMP_PHOS - cAMP and cGMP-dependent protein kinase phosphorylation site, ASN_GLYC - N-glycosylation site, Amidation - Amidation site, PTS-HPR - Serine Phosphorylation site signature, Rib S2 - Ribosomal protein S2 signature, SECA - SecA Family signature, ATP_HPR - ATP/GTP-binding site motif A, CPSASE 1 - Carbamoyl-phosphate synthase subdomain signature 1, CPSASE 2 - Carbamoyl-phosphate synthase subdomain signature 2, OMPA_1 - OMPA-like domain, GAPDH - Glyceraldehyde 3-phosphate dehydrogenase active site, RGD – Cell attachment sequence)

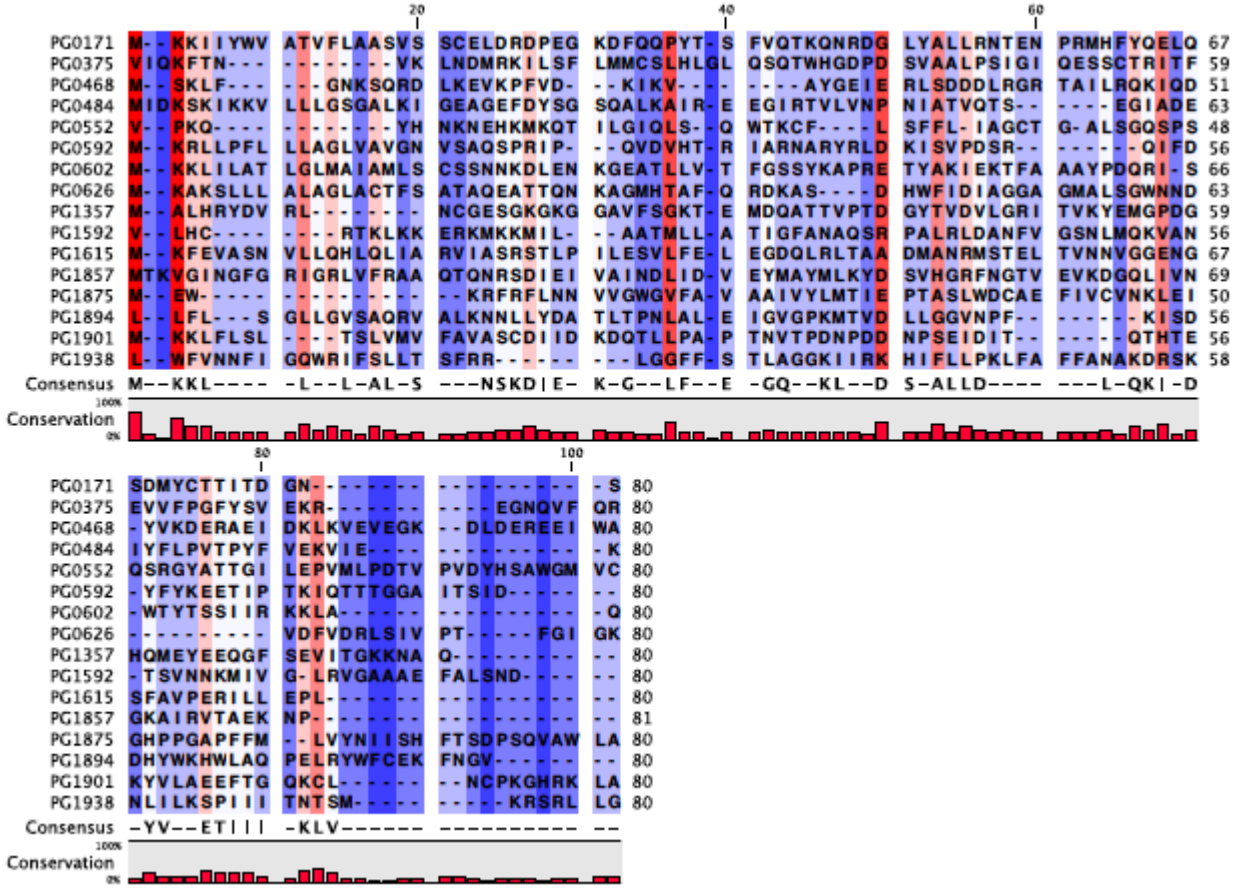


Fig. 3.4. Sequence alignment of the 15 missing extracellular proteins in FLL92 identified by Mass Spectrometric analysis. No unique sequence was common to all proteins.

## Peptidoglycan from W83 is Hydrolyzed Faster than FLL92

To determine whether the peptidoglycan of FLL92 was chemically dissimilar to that of FLL92, several lytic enzymes including lysozyme and lysostaphin were used (Fig 3.8). No significant change was observed between the wild-type and FLL92 using lysozyme; however, with the lysostaphin treated peptidoglycan, the rate of hydrolysis in W83 was twice that observed in FLL92 (lifetime of W83 - 270.47 ± 21.6; lifetime of FLL92 – 540.43 ± 82)

## The Murein Sacculi from FLL92 Differs from W83

TEM and AFM were used to determine the morphology and topography of the peptidoglycan Sacculi. The Sacculi from FLL92 when compared to the wild-type was distinctly different, as it possessed a homogeneously uneven surface with numerous contours. Sacculi from W83 were on average 100nm longer than those observed in FLL92 (Fig. 3.6 and Fig. 3.7.). Murein sacculi from both strains was subsequently treated with 15µg/ml of lysostaphin for ten minutes and imaged via AFM. Treated W83 were visibly different from the untreated control, as numerous contours were visible on the surface. In FLL92, the morphology of the treated samples was similar to the control.

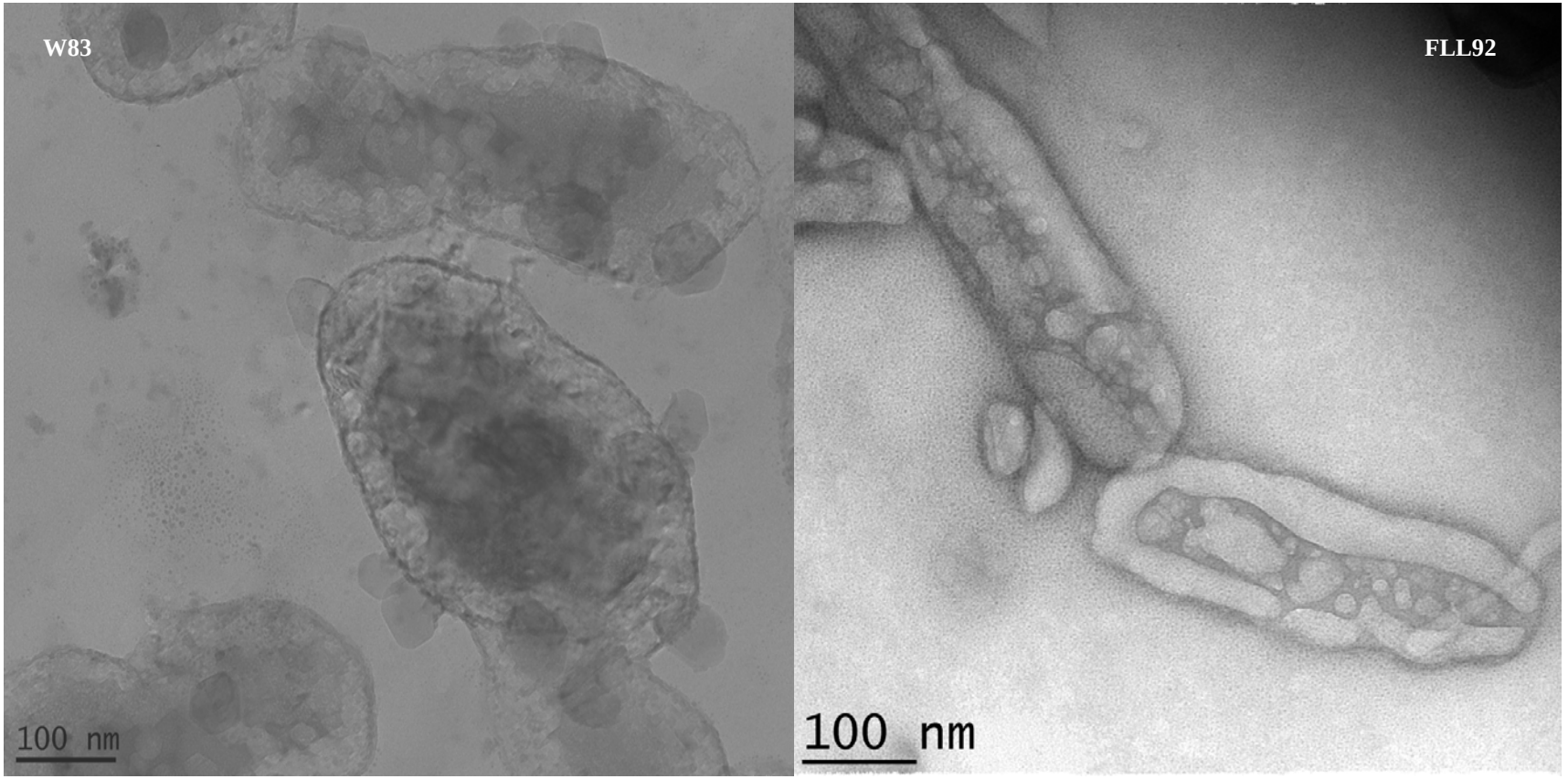


Fig. 3.5. Murein Sacculus Differs in FLL92 Compared to the wild-type

Transmission Electron Microscopy (TEM) was used to visualize the peptidoglycan sacculi of W83 and FLL92 strains, grown to late OD. Morphological differences were observed in FLL92, when compared to W83

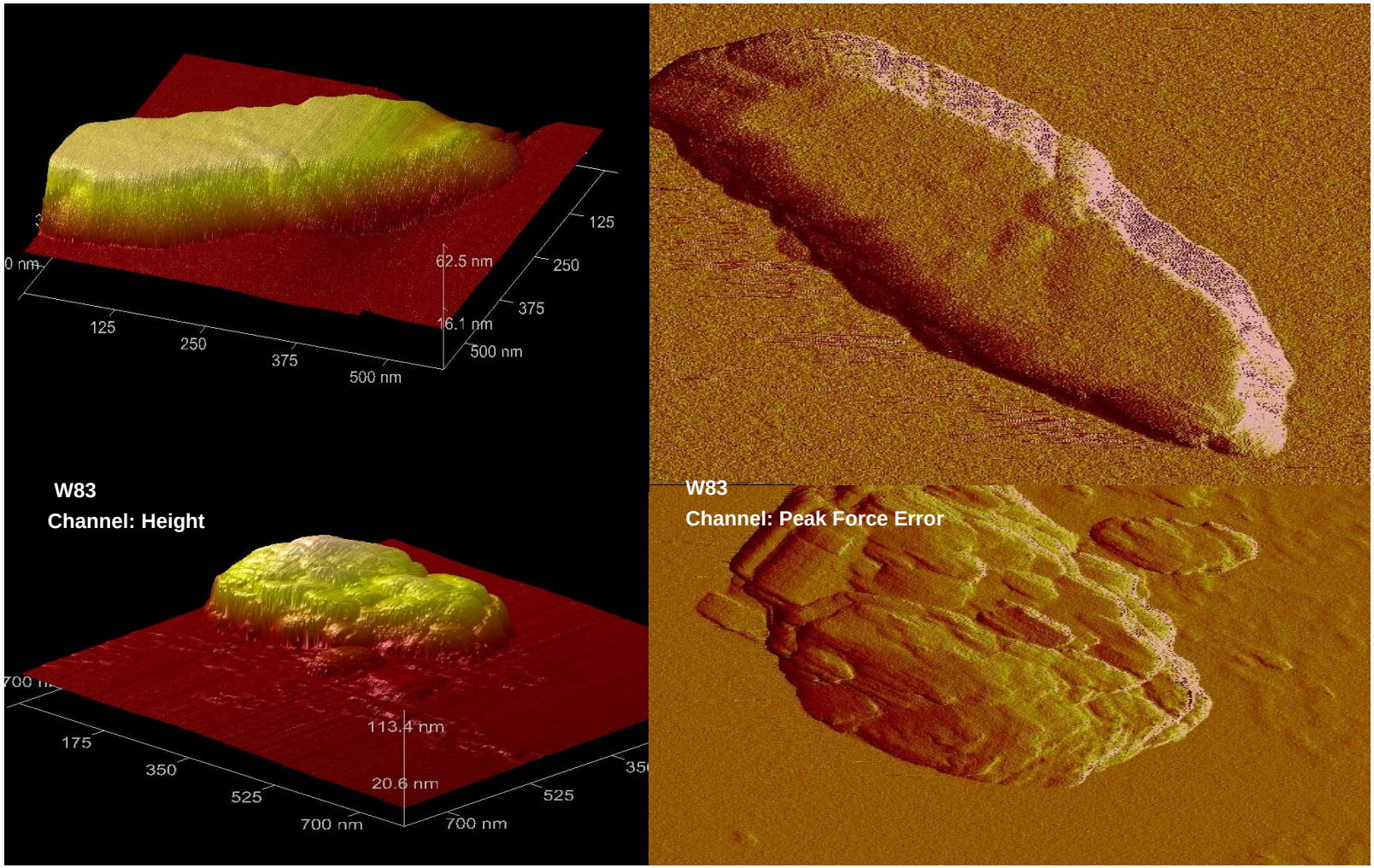


Fig. 3.6. Sacculi Topography of W83 and FLL92

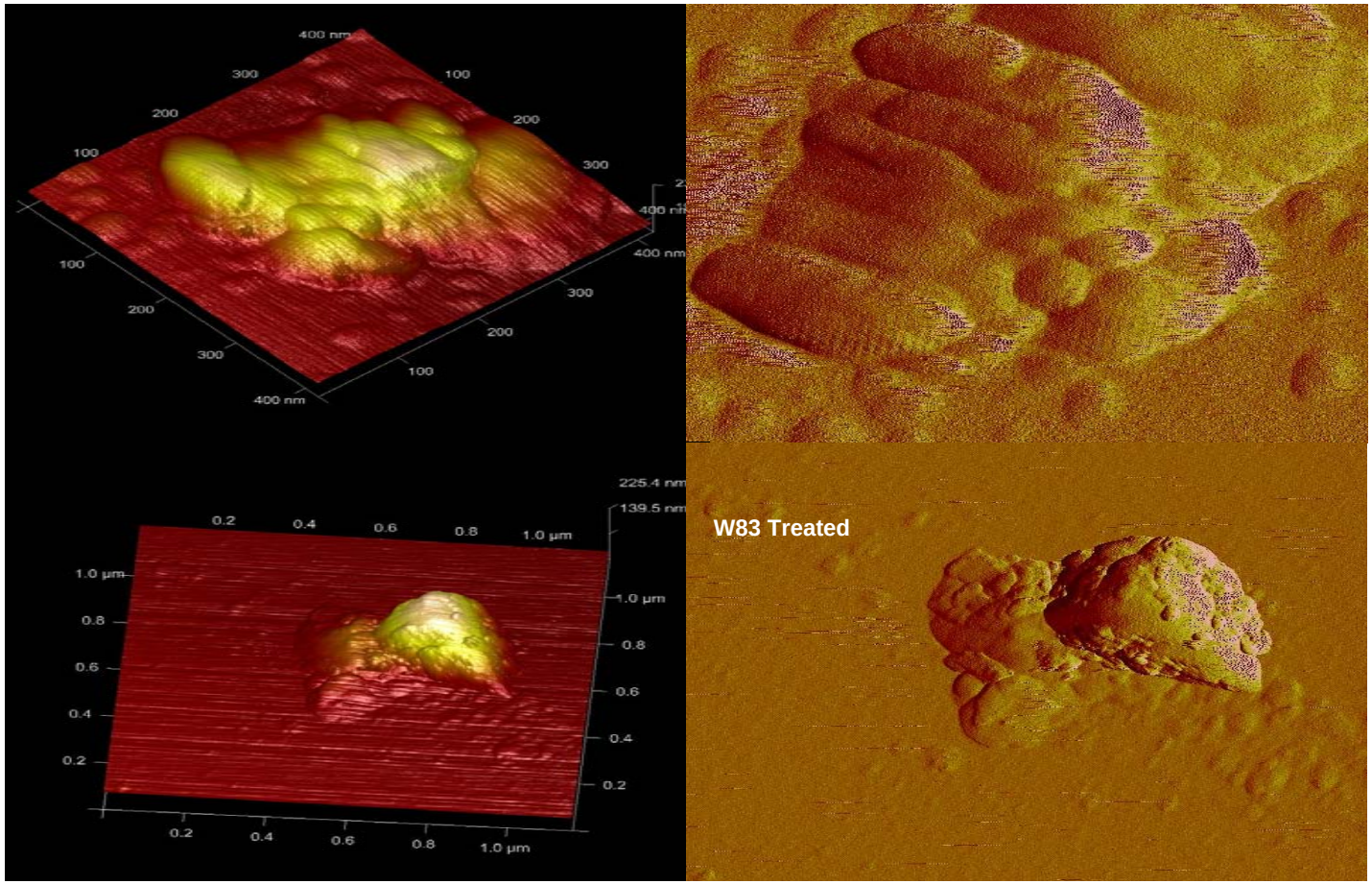


Fig. 3.7. Sacculi of W83 and FLL92, after 10min Treatment with 16µg/ml of Lysostaphin

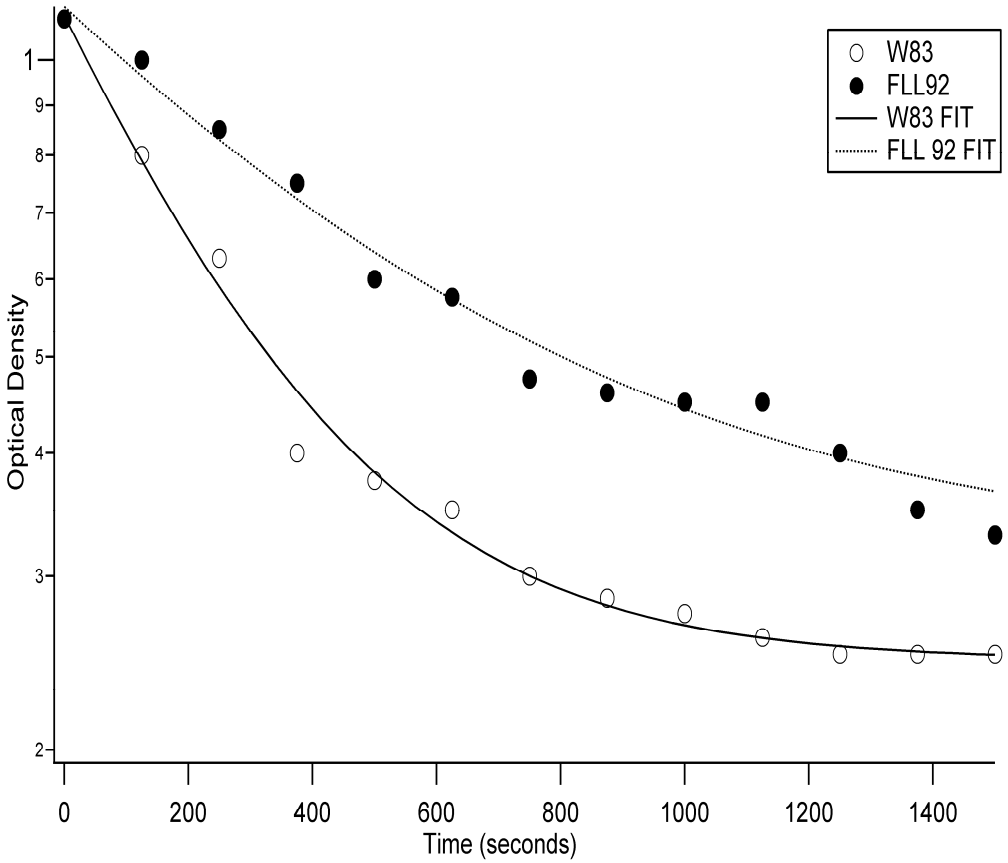


Fig. 3.8. Peptidoglycan from W83 is Hydrolyzed Twice as Fast as FLL92

## Discussion

In this study, we report that the peptidoglycan of FLL92 and the protein profile of the extracellular fraction are altered when compared to wild-type W83. VimA, a gene downstream of recA - encodes a 32 kDa protein. When inactivated, the phenotype of the mutant strain (FLL92) was non black pigmented, had reduced Arg-X and LysX specific proteolytic activity, lacked hemolytic and hemagglutinating activities, showed increased autoaggregation and was nonvirulent (1). Recently, the *vimA* gene was also shown to be involved in capsular and fimbrial biogenesis (33).

The phylogenetic tree comparing VimA to the FemABX proteins, suggests that FemX (0.45) is closer in relation to VimA (0.52) than the other Fem proteins. Members of the FemABX family are novel ribosomal Peptidyl transferases that are involved in the interchain peptide bridges of the peptidoglycan. Though primarily found in Gram positive bacteria they are observed in a few Gram negative bacteria, particularly the pathogenic spirochetes (13,15,15). FemX catalyzes the transfer of L-Ala onto the cytoplasmic UDP-N-acetylmuramyl-peptapetide (UDP-MurNAc-pentapetide) in contrast to the other Fem family members, which exclusively or preferentially transfers amino acids to membrane bound peptidoglycan precursors (28,36). The Kinetics of the FemX involves the sequential binding of UDP-MurNAc-pentapeptide and the Ala-tRNA to FemX, followed by the transfer of Ala to the ε-amino group of L-Lys in the pentapeptide stem and the subsequent release of the UDP-MurNAc-hexapeptide reaction products and the tRNA (36).

Several reports have indicated a role for the peptidoglycan in eliciting the host immune response (19,21); however, much remains unknown with regards the structural dynamics of the peptidoglycan in *P. gingivalis* and the enzymes involved in its biogenesis. Notably, there are no reported orthologs for the Fem family of proteins in *P. gingivalis*. The relative molar ratios of the glycan residues was previously reported to be

considerably lower in *P. gingivalis* when compared to *Bacteroides spp, Pseudomonas, Actinobaccillus* and *Actinomycetes,* suggestive of higher tetrapeptide to muramic acid ratios. The amino acid composition of this tetrapeptide contained Alanine, Diaminopimelicacid (DAP), Glycine and Lysine (17). Interestingly, previous studies in our lab have shown that recombinant VimA is able to interact with PG1101 (Alanyl t-RNA synthetase) (40). This finding is consistent with our *in silico* analysis, which suggests that VimA may have FemX function, as it would be expected to bind the Ala-tRNA and the UDP-MurNAc-pentapeptide. The conserved domains identified in both FemX and the VimA - RY*E**RO, KL****RDG****S, and EG*LL, may represent binding/interaction sites.

TEM and AFM microscopy showed morphological differences between W83 and FLL92. The sacculi from FLL92 was on average shorter in length than those observed in FLL92, and these sacculi also appeared granular in contrast to the smooth topography present in the wild-type. When wild-type sacculi were treated with lysostaphin (endopeptidase which specifically cleaves pentaglycine cross bridges) for ten minutes, the surface of W83 showed signs of stress with several contours indicative of hydrolysis, while the FLL92 sacculi showed no discernable change. In peptidoglycan assays using Lysozyme (catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D glucosamine) the percentage of hydrolysis was unchanged for the wild-type and mutant. Using lysostaphin, the rate of hydrolysis was two times that of FLL92, suggesting that the peptidoglycan from W83 is more efficiently hydrolyzed than in FLL92. It is likely, that the *vimA* dependent modification of the peptidoglycan may contribute to a structural modification, which may lead to its relative resistance to lysostaphin. In bacteria, o-acetylation of the peptidoglycan can modulate turnover by reducing the activity of autolysins and endogenous enzymes (7,23,34). The levels of o-acetylation in *P. gingivalis* FLL92 and W83 are unclear and the subject of further investigation.

In a previous report (33), we established that the VimA affects several surface related structures including fimbriae, capsule and some outer membrane proteins. To clarify whether this alteration in the protein profile was unique to the outer membrane or affected all secreted proteins, mass spectrometry was performed on the extracellular fraction. Sixty-eight proteins were identified as being aberrantly expressed in FLL92 (i.e. missing from the extracellular fraction of W83), this was markedly more than that observed in the outer membrane fraction (n=20). Interestingly, PG1496 - a conserved hypothetical protein predicted to be involved in transport, is aberrantly expressed in the outer membrane and extracellular fraction. Two other proteins with protein transport and binding functions – PG1497 and PG1504, were also aberrantly expressed. It is likely that these three proteins are part of a novel transporter/cell sorting system in *P. gingivalis,* which when disrupted, results in alterations in secretion and anchorage of proteins. Of note is the fact that several cytoplasmic proteins were found in the extracellular fraction of FLL92, including those involved in energy metabolism, translation and DNA metabolism. Though we cannot definitively rule out the possibility of cytoplasmic contamination, the fact that these protein fractions were all prepared under the same conditions using the same techniques, coupled with the fact that they are absent from the wild-type fraction, which showed no contamination with the periplasmic marker used – PG0535 (HtrA), leads us to believe that these proteins are incorrectly targeted for secretion in FLL92. PG1857 – Glyceraldehyde 3 phosphate dehydrogenase, which is involved in the colonization of *P. gingivalis* (30), is the only protein that was found to be missing from both the membrane and extracellular fractions of FLL92. An examination of proteins with varying spectral count values, suggest that Kgp is 6 times as abundant in W83 compared with FLL92, while RgpA has a negative fold change of 1.2. In a previous report, we have demonstrated that the gingipains though transcriptionally unaltered, are translationally affected by the *vimA* mutation, exemplified by the secretion of the inactive

precursor forms of these proteases (39,40). These results demonstrate that the disruption of the VimA – gingipain maturation pathway does not only lead to different forms being secreted, but also affects the concentration of these products. This phenomenon was also evident for the TonB dependent OM protein (PG0170), Carboxypeptidase D (PG0212), Hemmaglutinin (PG1602) and the Peptidylarginine deiminase (PG1249)

Several secreted proteins have been found to have primary and secondary structure similarity to the C-terminal domain of RgpB. These proteins have been designated the CTD family; and members of this family are attached to the cell surface through cell envelope glycans, which are important for proper folding and processing in order to produce a fully functional enzyme (37). *In silico* analysis was used to examine the C terminal of the missing extracellular proteins identified by mass spectrometry. We hypothesized that there would likely be a common motif unique to all these proteins; however, no common motif was present in missing proteins from the extracellular fraction, suggesting that a unique C terminal motif does not account for the alterations observed with the extracellular missing proteins.

Taken together, our results demonstrate that the VimA of *P. gingivalis* is needed for the correct secretion of several extracellular proteins and is likely involved in peptidoglycan biogenesis. This latter finding is the focus of ongoing research in our laboratory.

**Acknowledgements**

This work was supported by Loma Linda University and Public Health Grant DE13664 and DE019730 from NIDCR (to H.M.F).

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# CHAPTER FOUR

# DISCUSSION

*P. gingivalis* has several surface components that contribute to its virulence; these include - fimbriae, major outer membrane proteins, hemagglutinin, lipid polysaccharides, capsule and gingipains (5,24). Several genes - *vimA, vimE, vimF, porR, rfa* and *ugdA* (10,11,15,16,19-21) have been reported by our lab and others to be implicated in the formation of extracellular polysaccharides as well as glycan addition of gingipain-adhesin complexes (12). It nevertheless remains uncertain, whether the phenotypic change produced by mutations of these genes is due to the interruption of a central pathway, or multiple pathways.

The VimA which encodes a 32-kDa protein, was the first gene identified in our lab that showed the ability to modulate proteolytic activity. The phenotype of the *vimA* mutant strain (FLL92) when compared with the wild-type parent strain W83, was: non-black pigmented, non-virulent, had reduced hemagglutinating, hemolytic and proteolytic activity, showed increased autoaggregation and had a truncated LPS (10,21). The protein profile of FLL92 was also markedly different when compared to W83. Taken together, these results suggest that VimA plays a central role in the regulation and biogenesis of several surface structures and proteins. In this report, we address particular gaps in our understanding (Fig. 4.1.), regarding the role of VimA in capsular and fimbrial biogenesis, protein sorting and anchorage, acylation and glycosylation of outer membrane proteins, as well as its likely role in peptidoglycan biogenesis.

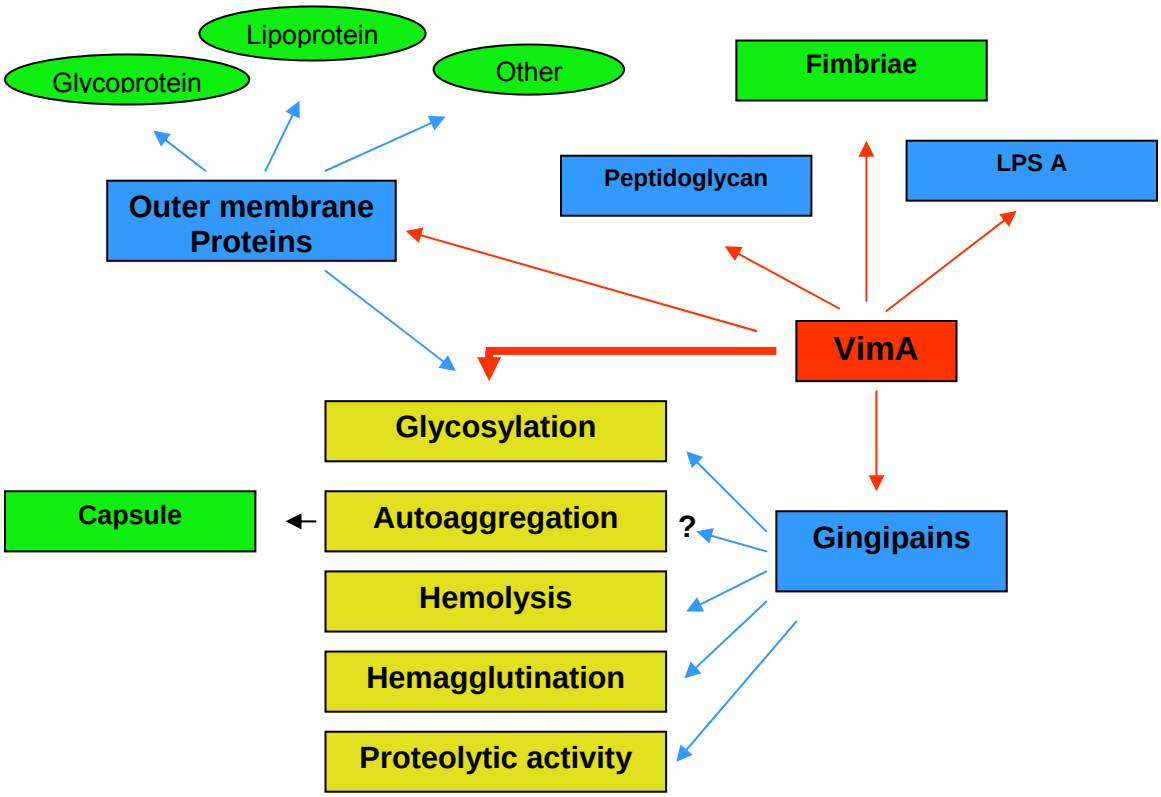


Fig. 4.1. Role of VimA

The VimA was predicted by in silico analysis to be closely related to the FemABX family of proteins. These proteins are found primarily in Gram positive bacteria and are involved in peptidoglycan biosynthesis (1,6,18). An examination of the phylogeny suggested that VimA (0.52) is closest in relation to the FemX (0.45). Notably, the FemX differs from the FemA and FemB which preferentially transfers amino acids to membrane bound peptidoglycan precursors, in that it catalyzes the transfer of L-Ala onto the cytoplasmic UDP-N-acetylmuramyl-peptapetide (UDP-MurNAc-pentapetide) (8,13). This process involves the sequential binding of Ala-tRNA and the UDP-MurNAc-pentapeptide. The ability of VimA to bind PG1101 (22), an Alanyl tRNA synthetase, further supports this function. Several attempts have been made to localize the VimA protein, using antibodies raised against the over expressed *E. coli* protein, this strategy has thus far proven unsuccessful, however there is evidence (unpublished) to indicate that this protein is likely cytoplasmic or periplasmic.

The role of VimA in peptidoglycan biogenesis was examined using TEM, AFM and hydrolytic enzyme assays. The peptidoglycan sacculi from FLL92 was distinctly different from that of W83, and had a granular appearance with numerous fine structures; additionally, peptidoglycan from FLL92 was hydrolyzed at a slower rate than W83. Taken together, our findings indicate a role for VimA in peptidoglycan biogenesis. Functional assays to demonstrate the ability of VimA to transfer Alanyl from a tRNA synthetase to the peptidoglycan are presently being pursued, as are experiments aimed at determining the difference in the peptide component of the peptidoglycan in FLL92 compared to W83.

Two separate superfamily prediction software yielded two likely classifications for the VimA. VimA was predicted to belong to the DUF482/CH1444 and/or the Acyl CoA N-acyltransferase superfamily. The DUF482/CH1444 family is part of the PEP-CTERM

system that has sortase like function (4) – involved in proteins sorting, while the Acyl CoA N-acyltransferase superfamily is a broad family which includes the FemABX nonribosomal peptidyltransferases family. The VimA was also predicted to interact with several proteins including RgpB, which is consistent with previous reports demonstrating the ability of VimA to interact with the gingipains (22).

Several reports have suggested that a correlation exists between fimbrial and capsular synthesis and the autoaggregation phenotype observed in *P. gingivalis* (3). This led us to hypothesize that the vimA mutation could effect capsular and fimbrial biogenesis. Transmission Electron Microscopy and Atomic Force Microscopy revealed that the capsule of FLL92 was irregular and fuzzy compared to the solid well defined capsule of the wild-type; additionally, FLL92 had a corrugated topography compared to with the smooth morphology present in W83. There was also increased fimbrial detection via immunogold staining and immunoblotting. Since the transcription level of fimA was unchanged in the mutant compared to the wild-type, hypothesize that the expression of capsular polysaccharides may impede the phenotypic expression of fimbriae, in a manner similar to that demonstrated in *Klebsiella pneumoniae* (7,14), i.e. direct physical interaction might impede the polymerization of fimbriae on the cell surface (Fig. 4.2.). These findings are in contradiction to a recent report which suggests, that the inability of W83 to produce fimbriae is due to a mutated FimSR histidine kinase and the inability of W83 FimA to polymerize on the outer membrane of the cell (9). Our findings suggest that an alternate FimSR independent pathway may exist for fimbrial maturation. Further studies are needed to determine whether the VimA plays a direct role in the synthesis and regulation of these two macro-structures, and the degree of FimA polymerization in the mutant.

Mass spectrometry was performed on proteins from the membrane and extracellular fractions of FLL92, in order to determine the types of proteins that were

being affected by the VimA mutation, and to elucidate whether the VimA could have sortase-like function. In the membrane fraction of FLL92, twenty proteins were identified as aberrantly anchored to the cell wall of FLL92, while nine proteins were missing. Twenty proteins had variable spectral count values (normalized spectral count values correspond to the relative abundance of the protein). In the extracellular fraction, sixty eight proteins were identified as aberrantly expressed, while fifteen proteins were missing and 20 had variable spectral count values.

The absence of Carboxypeptidase D on the outer membrane of FLL92 likely explains why Kgp and RgpA are not anchored to the outer membrane, as these enzymes require C terminal processing (2,23). Interestingly, this enzyme is present at lower abundance in the extracellular fraction of FLL92 Compared with W83 - negative fold change of 28. Several transport proteins and several structural membrane proteins are aberrantly expressed or missing in this mutant. It is likely, that the absence of these transport and structural proteins result in a disruption in the normal cell sorting and secretion machinery, resulting in incorrect and unregulated protein secretion. Mass spectrometry also confirmed our previous finding (21,22) that RgpA and Kgp are only found in the extracellular fraction of FLL92; notably however, these gingipains are at much lower levels of abundance. RgpA showed a negative fold change of 1.2, while Kgp had a negative fold change of 6.6. Taken together, these findings support a role for VimA in anchorage and maturation of outer membrane proteins and the correct targeting of secreted proteins. The interaction between VimA and the missing or aberrantly expressed outer membrane and extracellular proteins, is an active area of investigation.

Previous reports in our lab have highlighted the aberrant protein glycosylation that occurs in FLL92 (21); however, not much is known about the particular carbohydrate moieties involved in this aberrant glycosylation profile. Lectin binding assays allowed for the identification of these moieties. Outer membrane proteins glycosylated with

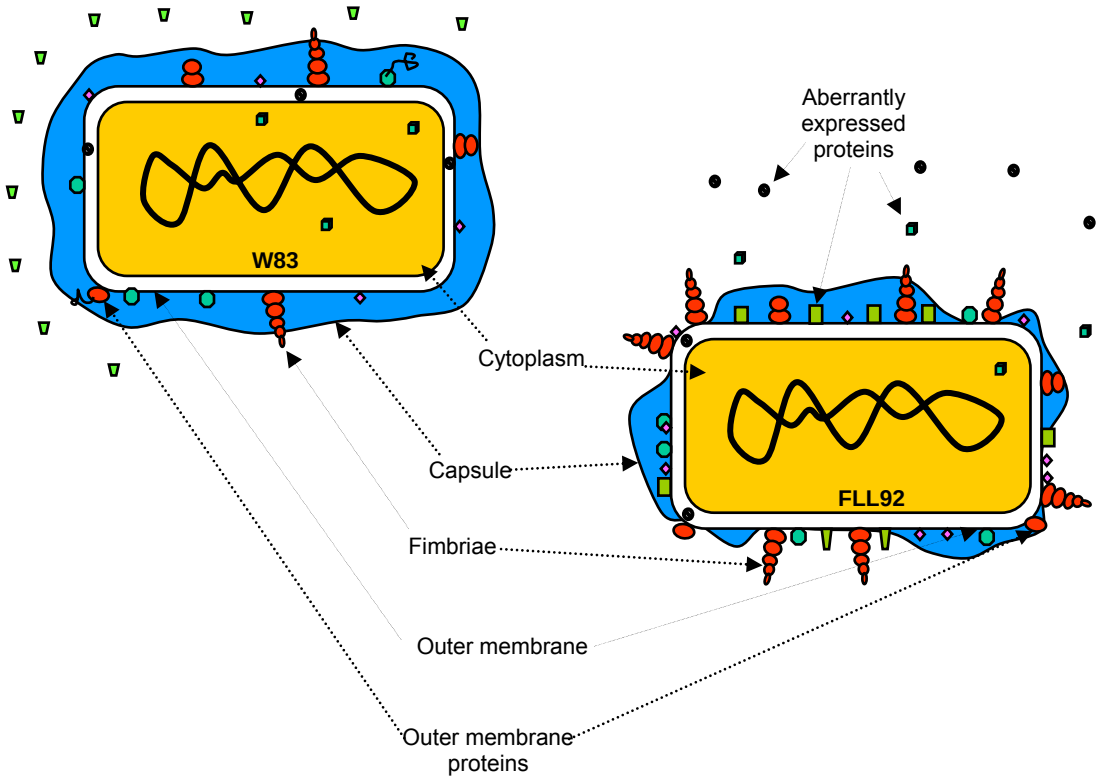


Fig. 4.2. Ultrastructural variations modulated by VimA

Galactose (β 1,3) N-Acetylgalactosamine, N-acetyl-α-D-galactosamine, Galactose (β 1,4) N-Acetylglucosamine, N-acetyl-D-galactosamine and Sialic Acid (N-Acetyl neuramic acid) were affected by the *vimA* mutation. These findings indicate a role for VimA in the correct glycosylation of membrane proteins.

Lipid modification of outer-membrane proteins in *P. gingivalis* has been well characterized (17), based on the characterization of VimA (A putative acyl transferase), we hypothesized that it would play a role in the acylation of one or more membrane proteins. Using $^{3}H$ labeled palmitic acid as the lipid donor, the role of VimA was examined. No changes were observed in the lipid modified protein profile of the wild-type total protein fraction. Comparatively, distinct differences were observed in the extracellular fraction of FLL92 when compared with W83. A 54 kDa protein was three times more abundant in W83 than in FLL92, while a 27 kDA protein was twice as abundant in FLL92 compared with W83. These results suggest that the VimA is important for the correct acylation of a 54 kDa protein in the extracellular fraction, or that acylation may be required for dimerization of the 27 kDa protein. Several studies are presently ongoing with a view to clarify this observation.

Our findings support the view that VimA is a multifunctional – multi domain protein, involved in the regulation of membrane components including – fimbriae, capsule, and peptidoglycan. This protein is also involved in the sorting and secretion of several proteins and plays a role in the correct glycosylation of several outer membrane proteins and the correct acylation of at least two secreted proteins. Our results indicate a putative central role for the VimA protein in membrane biogenesis, protein modification and transport. These putative multiple functions require further elucidation.

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